WEST Search History

Hide Items | Restore | Clear | Cancel

DATE: Wednesday, April 26, 2006

Hide?	Set Name	Query	Hit Count
		DB=PGPB,USPT,USOC,EPAB,JPAB,DWPI,TDBD; PLUR=YES; OP=OR	
☐	L1	kinase near100 complex	5781
☐	L2	kinase near10 complex	4683
☐	L3	L2 same (immobil$ or attach$ or solid or surface or bead or heterologous or well or sensor or tube or container)	1455
☐	L4	L2 near50 (immobil$ or attach$ or solid or surface or bead or heterologous or well or sensor or tube or container)	600
☐	L5	L4 and (posttranslation$ or post-translation or (covalent near2 modification) or phophorylat$ or dephosphorylat$ or acylat$ or deacylat$ or glycosylat$ or deglycosylat$ or ubiquitinat$ or deubiquitinat$ or prenylat$ or deprenylat$ or sentrinizat$ or sentrinisat$ or desentrinizat$ or desentrinisat$ or ribosylat$ or deribosylat$)	481
☐	L6	L4 same (posttranslation$ or post-translation or (covalent near2 modification) or phophorylat$ or dephosphorylat$ or acylat$ or deacylat$ or glycosylat$ or deglycosylat$ or ubiquitinat$ or deubiquitinat$ or prenylat$ or deprenylat$ or sentrinizat$ or sentrinisat$ or desentrinizat$ or desentrinisat$ or ribosylat$ or deribosylat$)	9
☐	L7	pyk2 or pyk-2 or pky2 or pky-2	397
☐	L8	L7 and immobili$.ti,ab,clm.	7
☐	L9	L7.ti,ab,clm.	63
☐	L10	L9 not l9	0
☐	L11	L9 not l8	62

END OF SEARCH HISTORY

C07D235:00 C07D471/04.

☐ 56. WO 9835056A. Identifying agents that bind and modulate protein tyrosine kinase 2 - useful for inhibiting migration, adhesion or activity of monocytic cells, particularly for treatment and prevention of osteoporosis and inflammation. DUONG, L T, et al. A61K038/00 A61K038/17 A61K045/00 A61P019/10 A61P029/00 A61P043/00 C07K014/00 C12N009/12 C12N015/09 C12N015/52 C12Q001/48.

☐ 57. WO 9835016A. New nucleic acid encoding murine protein tyrosine kinase 2 and cells expressing the recombinant kinase - used to identify specific modulators, potentially useful for controlling the level of platelets. DUONG, L T, et al. C12N001/00 C12N001/15 C12N001/19 C12N001/21 C12N005/10 C12N009/12 C12N015/09 C12N015/54 C12Q001/48 C12Q001/68 C12N009/12 C12R001:91.

☐ 58. US20020048782A. New indolinone(s), that bind to, and modulate, PYK2 tyrosine kinase - useful for, e.g. treating and diagnosing epilepsy, stroke, schizophrenia and hyperactivity in children. LEV, S, et al. A61K031/421 A61K031/47 A61K031/498 A61K031/517 C07H021/04 C07K001/00 C07K014/00 C07K017/00 C12N001/19 C12N001/21 C12N005/00 C12N005/02 C12N005/06 C12N005/10 C12N009/12 C12N015/09 C12N015/12 C12N015/63 C12P021/02 C12P021/06 C12Q001/68 G01N033/53.

☐ 59. EP 931146A. New nucleic acid encoding human retinal degradation polypeptide - and related probes, vectors, transformed cells, proteins and antibodies, used to diagnose neurological disease and to identify specific modulators with possible therapeutic activity. LEV, S, et al. A61K038/17 C07H021/04 C07K014/435 C07K016/18 C12N001/20 C12N005/12 C12N015/00 C12N015/12 C12N015/63 C12P021/06.

☐ 60. US 5837524A. Probin tyrosine kinase 2 and nucleic acid encoding PYK2 - used to detect and treat diseases characterised by a signal transduction pathway abnormality, e.g. epilepsy or schizophrenia. LEV, S, et al. A23J001/00 A61K045/00 A61P025/04 A61P025/06 A61P025/08 A61P025/18 A61P025/28 C07C255/34 C07D215/00 C07D239/72 C07D241/40 C07H021/04 C07K014/00 C07K016/40 C12N001/21 C12N005/06 C12N005/12 C12N009/12 C12N015/09 C12N015/54 C12N015/64 C12P012/06 C12P021/02 C12Q001/48 C12Q001/68 G01N033/574 G01N033/68.

☐ 61. JP 07067630A. Prepn. of monoclonal antibody-producing hybridoma for cancer diagnosis and treatment - using auto-immune diseased animal to produce hybridoma specifically recognising N-glucosyl-neuraminic acid contg. saccharide chain. A61K039/395 C07K016/46 C12N005/10 C12N015/02 C12P021/08 C12P021/08 C12R001:91 C12P021/08 C12R001:91.

☐ 62. JP 62235569A. Monoclonal antibody - used in cancer studies and has specific recognition for saccharide contg. N-glycol:yl neuramine. A61K039/39 A61K039/395 C07K015/04 C12N005/00 C12N015/06 C12P021/00 C12P021/08 C12R001/91 G01N033/57 G01N033/574 G01N033/577 C12P021/08 C12R001:91.

Generate Collection | Print

Term	Documents
(9 NOT 8).PGPB,USPT,USOC,EPAB,JPAB,DWPI,TDBD.	62
(L9 NOT L8).PGPB,USPT,USOC,EPAB,JPAB,DWPI,TDBD.	62

Generate Collection | Print

Search Results - Record(s) 1 through 50 of 62 returned.

☐ 1. 20060029546. 06 Aug 04. 09 Feb 06. Materials and methods for identifying anti-schizophrenic agents. Gurney; Mark E., et al. 424/9.1; 435/6 C12Q1/68 20060101 A61K49/00 20060101

☐ 2. 20050222171. 20 Jan 05. 06 Oct 05. Organic compounds. Bold, Guido, et al. 514/259.3; 544/281 A61K031/519 C07D487/04.

☐ 3. 20050170431. 27 Feb 04. 04 Aug 05. PYK2 crystal structure and uses. Ibrahim, Prabha, et al. 435/7.1; 530/388.26 G01N033/53 C07K016/40.

☐ 4. 20050164300. 15 Sep 04. 28 Jul 05. Molecular scaffolds for kinase ligand development. Artis, Dean R., et al. 435/7.1; 702/19 G01N033/53 G06F019/00 G01N033/48 G01N033/50.

☐ 5. 20050059621. 04 Jun 04. 17 Mar 05. Methods for using modulators of proline-rich tyrosine kinase 2. Griswold-Prenner, Irene, et al. 514/44; 514/249 514/266.1 514/312 A61K048/00 A61K031/517 A61K031/47.

☐ 6. 20040259158. 27 Feb 04. 23 Dec 04. PYK2 and inflammation. Schlessinger, Joseph, et al. 435/7.1; 514/266.1 514/312 514/418 G01N033/53 A61K031/517 A61K031/47.

☐ 7. 20040197332. 09 Feb 04. 07 Oct 04. Inhibitors of her3 activity. Ullrich, Axel, et al. 424/145.1; A61K039/395.

☐ 8. 20040186157. 12 Jan 04. 23 Sep 04. Cyclic substituted fused pyrrolocarbazoles and isoindolones. Hudkins, Robert L., et al. 514/410; 548/418 A61K031/407 C07D209/56.

☐ 9. 20040127453. 26 Jul 02. 01 Jul 04. Method for treating diseases associated with abnormal kinase activity. Lyons, John, et al. 514/50; A61K031/7072.

☐ 10. 20040082510. 31 Jul 03. 29 Apr 04. Pyk2 phosphorylation by her3 induces tumor invasion. Ullrich, Axel, et al. 514/12; A61K038/17.

☐ 11. 20040072836. 23 Sep 03. 15 Apr 04. Substituted aza-oxindole derivatives. Harris, Philip Anthony, et al. 514/249; 514/265.1 514/300 544/281 544/350 546/113 C07D487/02 C07D471/02 A61K031/519 A61K031/498.

☐ 12. 20040072266. 23 Sep 02. 15 Apr 04. Method of modifying cell motility via compounds and polypeptides that interact with a Wnt pathway. Wilder, Elizabeth L., et al. 435/7.23; 435/455 G01N033/574 C12N015/85.

☐ 13. 20040053924. 21 Jul 03. 18 Mar 04. 4-Substituted 7-aza-indolin-2-ones and their use as protein kinase inhibitors. Liang, Congxin, et al. 514/234.5; 514/252.16 514/253.04 514/265.1 514/300 544/117 544/127 544/281 544/362 546/113 A61K031/5377 A61K031/519 A61K031/496 A61K031/4745 C07D471/02.

☐ 14. 20040048816. 24 Feb 03. 11 Mar 04. Restenosis treatment. Zohlnhofer, Dietlind, et al. 514/44; 424/145.1 604/500 A61K048/00 A61K039/395 A61M031/00.

☐ 15. 20040029124. 13 Feb 03. 12 Feb 04. Mrna amplification. Zohlnhofer, Dietlind, et al. 435/6; 435/91.2 C12Q001/68 C12P019/34.

☐ 16. 20040005648. 19 Jun 03. 08 Jan 04. PYK2 related products and methods. Lev, Sima, et al. 435/7.23; 435/194 435/320.1 435/338 435/69.1 530/388.26 536/23.2 G01N033/574 C07H021/04 C12N009/12 C12P021/02 C12N005/06.

☐ 17. 20030190688. 07 Apr 03. 09 Oct 03. Methods for detecting BCR-ABL signaling activity in tissues using phospho-specific antibodies. Crosby, Katherine, et al. 435/7.23; G01N033/574.

☐ 18. 20030119067. 13 Nov 02. 26 Jun 03. PYK2 related products and methods. Lev, Sima, et al. 435/7.1; 435/194 435/320.1 435/325 435/69.1 514/249 514/266.1 514/311 514/376 G01N033/53 C12N009/12 C12P021/02 C12N005/06 A61K031/498 A61K031/517 A61K031/421 A61K031/47.

☐ 19. 20030069430. 30 Nov 01. 10 Apr 03. Substituted oxindole derivatives as protein tyrosine and as protein serine/threonine kinase inhibitors and compositions and methods of treating chemotherapy and radiation therapy side effects. Davis, Stephen Thomas, et al. 546/277.7; 548/486 C07D41/02 C07D209/34.

☐ 20. 20020183319. 21 Dec 00. 05 Dec 02. 4-substituted 7-aza-indolin-2-ones and their use as protein kinase inhibitors. Liang, Congxin, et al. 514/234.5; 514/252.16 514/265.1 514/300 544/117 544/127 544/280 544/362 546/113 A61K031/5377 A61K031/519 A61K031/496 A61K031/4745 C07D471/02 C07D491/02.

☐ 21. 20020048782. 09 Dec 97. 25 Apr 02. PYK2 RELATED PRODUCTS AND METHODS. LEV, SIMA, et al. 435/69.1; 435/325 435/7.1 530/350 536/23.5 G01N033/53 C12Q001/68 C07H021/04 C12P021/06 C12N005/00 C12N005/02 C07K001/00 C07K014/00 C07K017/00.

☐ 22. 20020045191. 17 Sep 01. 18 Apr 02. Inhibition of the SRC kinase family pathway as a method of treating HBV infection and hepatocellular carcinoma. Schneider, Robert J., et al. 435/7.1; 435/15 514/262.1 514/44 514/520 514/7 536/24.5 A61K048/00 A61K038/17 A61K031/519 A61K031/277 A01N037/34 A61K031/275 A01N043/04 A61K031/70 C07H021/04 C12Q001/48 G01N033/53 A61K038/16.

☐ 23. 6861442. 30 Dec 99; 01 Mar 05. PYK2 and inflammation. Schlessinger; Joseph, et al. 514/414; 424/94.1 435/15 435/7.4 514/227.5 514/229.8 514/235.8 514/311 514/312 514/418 514/419 514/450 514/514 514/546. A01N043/38 .

☐ 24. 6610688. 21 Dec 00; 26 Aug 03. 4-substituted 7-aza-indolin-2-ones and their use as protein kinase inhibitors. Liang; Congxin, et al. 514/234.2; 514/234.5 514/252.16 514/265.1 544/117 544/280. C07D487/04 A61K031/519 .

☐ 25. 6576442. 07 Jun 00; 10 Jun 03. rdgB proteins and related products and methods. Lev; Sima, et al. 435/69.1; 435/252.3 435/320.1 530/300 530/350 536/23.1 536/23.5. C12N015/00 C12N001/20 C12P021/06 C07H021/04 .

☐ 26. 6117673. 26 Sep 97; 12 Sep 00. RDGB proteins and related products and methods. Lev; Sima, et al. 435/320.1; 435/252.3 435/69.1 530/350 536/23.1 536/23.5. C12N015/00 C12N001/20 C12P021/06 C07H021/04 .

☐ 27. 5837815. 02 Jun 95; 17 Nov 98. PYK2 related polypeptide products. Lev; Sima, et al. 530/350; 435/69.1 530/412. C12P012/06 C07K014/00 A23J001/00 .

☐ 28. 5837524. 15 Dec 94; 17 Nov 98. PYK2 related polynucleotide products. Schlessinger; Joseph, et al. 435/252.3; 435/320.1 435/91.4 536/23.1 536/25.4. C12N015/64 C12N001/21 C07H021/04 .

☐ 29. 4965198. 26 Apr 88; 23 Oct 90. Monoclonal antibody and method of manufacturing hybridoma producing the same. Yamasaki; Masahiko, et al. 530/387.5; 435/329 435/344.1 435/452 435/70.21 530/388.85 530/861 530/864. C12N005/00 C12P021/00 .

☐ 30. WO2005002573A2. 04 Jun 04. 13 Jan 05. METHODS FOR USING MODULATORS OF PROLINE-RICH TYROSINE KINASE 2. GRISWOLD-PRENNER, IRENE, et al. A61K031/41;.

☐ 31. WO2004078923A2. 27 Feb 04. 16 Sep 04. PYK2 CRYSTAL STRUCTURE AND USES. IBRAHIM, PRABBA, et al. C12N00/;.

☐ 32. EP001361229A2. 06 Dec 95. 12 Nov 03. Use of Tyrosine Kinase (PYK2) to produce antibodies. LEV, SIMA DR, et al. C12N015/54; C12N009/12 C12Q001/68 C07K016/40 C12N005/12 G01N033/68 C12Q001/48 C07D241/40 C07C255/34 C07D215/00 C07D239/72.

☐ 33. EP001228766A1. 31 Jan 01. 07 Aug 02. PYK2 phosphorylation by HER3 induces tumor invasion. A61K038/17; A61K038/45 A61K039/395 G01N033/68.

☐ 34. WO009835016A1. 09 Feb 98. 13 Aug 98. PROTEIN TYROSINE KINASE 2 (PYK2), NUCLEIC ACIDS, AND ASSAY. DUONG, LE T, et al. C12N001/00; C12N005/10 C12N015/54 C12Q001/48 C12Q001/68.

☐ 35. WO009826054A2. 09 Dec 97. 18 Jun 98. PYK2 RELATED PRODUCTS AND METHODS. LEV, SIMMA, et al. C12N009/12; C12N015/12 C12N015/63 C12N001/21 C12N001/19 C12N005/10.

☐ 36. WO009618738A2. 06 Dec 95. 20 Jun 96. PROBIN TYROSINE KINASE (PYK2) ITS cDNA CLONING AND ITS USES. LEV, SIMA, et al. C12N015/54; C12N009/12 C12Q001/68 C07K016/40 C12N005/12 G01N033/68 C12Q001/48 C07D041/40 C07C255/34 C07D215/00 C07D239/72.

☐ 37. US20060029546A. Identifying N-methyl-D-Aspartate (NMDA) receptor function modulator, involves administering test agent to mammal harboring genetic defect in neuregulin 1 signaling pathway, and measuring NMDA receptor complex tyrosine phosphorylation. ANDRESSEN, T, et al. A61K049/00 C12Q001/68 G01N033/50.

☐ 38. EP 1621539A. New heterocycle-substituted cyclic urea derivatives useful as protein tyrosine kinase inhibitors for treating e.g. fibrotic disorders, acromegaly, allergies, asthma, Crohn's disease and retinopathy. GUESSREGEN, S, et al. A61K031/4164 A61K031/4178 A61K031/513 A61K031/551 C07D401/00 C07D401/14 C07D405/00 C07D405/14 C07D413/00 C07D413/14 C07D417/00 C07D417/14.

☐ 39. EP 1621536A. New amino cyclic urea derivatives are protein tyrosine kinase inhibitors, useful to treat/prevent e.g. disorders of blood vessel proliferation, fibrotic disorders, disorders of mesangial cell proliferation and acromegaly. BENARD, D, et al. A61K000/00 C07D401/00 C07D401/06 C07D401/14.

☐ 40. US20050222171A. New pyrazolo(1,5-a)pyrimidin-7-ylamine compounds, useful to treat e.g. benign tumor, psoriasis, prostate hyperplasia, Crohn's disease, Hodgkin's disease, leukemia and rheumatoid arthritis, are protein kinase inhibitors. BOLD, G, et al. A61K031/519 C07D487/04.

☐ 41. WO2005002573A. Preventing cell death in neuron, involves administering non-receptor proline-rich tyrosine kinase inhibitor composition to neuron, useful for treating Alzheimer's disease, stroke or Parkinson's disease. GRISWOLD-PRENNER, I, et al. A61K031/41 A61K031/47 A61K031/517 A61K048/00.

☐ 42. WO2004078923A. Crystalline form of kinase domain of protein tyrosine kinase 2 (PYK2), useful for identifying potential PYK2 binding compounds that are useful in treating PYK2 associated diseases such as cancer or inflammation. IBRAHIM, P, et al. C07K016/40 C12N000/00 C12N001/00 G01N033/53.

☐ 43. US20030190688A. Detection of activity of kinase and/or signaling pathway in cell or tissue involves contacting test cell or tissue with phospho-specific antibody(s). CROSBY, K, et al. G01N000/00 G01N033/574.

☐ 44. WO2003020698A. New substituted-2-aryl benzimidazole and benzofuran(3,2-c)quinoline compounds useful in the treatment of e.g. bone and cartilage related disorders, are protein tyrosine inhibitors. BOGIN, O, et al. A61K031/4184 C07D000/00 C07D235/18.

☐ 45. WO 200276985A. New kinase inhibitor useful e.g. for treating rheumatoid arthritis, psoriasis. BONDINELL, W E, et al. A61K031/4375 A61K031/4985 C07D487/04.

☐ 46. EP 1228766A. Use of HER3 or PYK2 protein, a nucleic acid encoding HER3 or PYK protein, or its complement, as a target for modulation of mitogen-activated protein kinase pathway. ULLRICH, A, et al. A61K031/7052 A61K038/00 A61K038/17 A61K038/45 A61K039/395 A61P029/00 A61P035/00 A61P035/02 A61P043/00 C12Q001/48 G01N033/68.

☐ 47. US20020045191A. Treating Hepatitis B virus infection and hepatocellular carcinoma, by administering a compound that modulates the synthesis or expression of a target cellular gene or the activity of a target protein, e.g. Pyk2 kinase. KLEIN, N, et al. A01N037/34 A01N043/04 A61K031/275 A61K031/277 A61K031/519 A61K031/70 A61K038/16 A61K038/17 A61K048/00 C07H021/04 C12Q001/48 G01N033/53.

☐ 48. US20020052369A. New indolinone derivatives, useful as modulators of protein kinases and phosphatases for treating e.g. cancer, and inflammatory, immunological and cardiovascular disorders. HARRIS, G D, et al. A61K031/404 A61K031/4045 A61K031/4439 A61K031/4709 A61K031/4725 A61K031/5377 A61P003/10 A61P009/00 A61P017/06 A61P019/02 A61P029/00 A61P035/00 A61P037/02 A61P037/06 A61P043/00 C07D209/34 C07D401/14 C07D403/02 C07D403/14 C07D413/02 C07D413/14 G01N033/53.

☐ 49. WO 200170953A. Use of an inhibitor of the interferon-gamma signaling pathway for preparation of a pharmaceutical composition useful in the treatment or prevention of restenosis. BAEUERLE, P, et al. A61K031/00 A61K031/417 A61K031/7088 A61K038/00 A61K039/395 A61K045/00 A61K048/00 A61M031/00 A61P009/00 A61P009/08 A61P009/10 A61P043/00 C12N009/00 C12N015/11 C12Q001/02 C12Q001/68 G01N033/15 G01N033/50.

☐ 50. WO 200056709A. New indolinone derivatives are protein kinase inhibitors used for treating e.g. cardiovascular, dermatological and immune disorders and cancer. HARRIS, G D, et al. A61K031/40

A61K031/404 A61K031/4439 A61K031/5377 A61P003/10 A61P009/00 A61P009/10 A61P017/06 A61P019/02 A61P029/00 A61P035/00 A61P037/00 A61P043/00 C07D209/34 C07D209:00 C07D401/04 C07D401/06 C07D401/10 C07D401/12 C07D401/14 C07D403/06 C07D403/10 C07D409/04 C07D409/10 C07D409/14 C07D413/12 C07D413/14 C12N009/99 C07D213:00 C07D401/12 C07D213:00 C07D401/06 C07D333:00 C07D409/10 C07D409/10 C07D401/12 C07D401/06 C07D333:00 C07D213:00 C07D213:00 C07D209:00 C07D209:00 C07D209:00.

Generate Collection Print

Term	Documents
(9 NOT 8).PGPB,USPT,USOC,EPAB,JPAB,DWPI,TDBD.	62
(L9 NOT L8).PGPB,USPT,USOC,EPAB,JPAB,DWPI,TDBD.	62

Prev Page Next Page Go to Doc#

Generate Collection | Print

Search Results - Record(s) 51 through 62 of 62 returned.

☐ 51. WO 200049144A. Pyk2 binding protein nucleic acids, polypeptides, antibodies and antisense oligonucleotides, the oligonucleotides are useful for treating cancer or a cardiovascular, neurodegenerative or immune disorder. CHEN, A, et al. A01K067/027 A61K031/7084 A61K045/00 A61K048/00 A61P003/10 A61P009/00 A61P013/12 A61P017/02 A61P017/06 A61P019/04 A61P025/00 A61P029/00 A61P035/00 A61P037/00 A61P043/00 C07H021/00 C07K014/47 C07K016/18 C12N001/15 C12N001/19 C12N001/21 C12N005/10 C12N015/02 C12N015/09 C12N015/11 C12N015/12 C12Q001/02 C12Q001/68 G01N033/15 G01N033/50 G01N033/53 G01N033/566.

☐ 52. WO 200040971A. Identifying compounds for treating diseases/disorders characterized by an inflammatory response such as inflammatory bowel diseases, comprising assaying for modulators of the interaction between PYK2 polypeptide and a binding partner. GISHIZKY, M, et al. A01N043/38 A61K031/343 A61K031/47 A61K031/498 A61K031/517 A61K031/661 A61K045/00 A61P001/00 A61P001/02 A61P001/04 A61P007/00 A61P019/02 A61P019/04 A61P021/04 A61P027/02 A61P029/00 C12N009/12 C12Q001/48 G01N033/15 G01N033/50 G01N033/53 G01N033/566 G01N033/573 G01N033/68 G01N033/86.

☐ 53. WO 200012084A. New 2-indolinone derivatives useful for treating cancer, autoimmune disorders and restenosis. MILLER, T A, et al. A61K031/34 A61K031/35 A61K031/36 A61K031/38 A61K031/385 A61K031/39 A61K031/40 A61K031/404 A61K031/405 A61K031/41 A61K031/415 A61K031/42 A61K031/425 A61K031/435 A61K031/44 A61K031/495 A61K031/50 A61K031/505 A61K031/517 A61K031/519 A61K031/53 A61K031/535 A61K031/54 A61P003/10 A61P007/00 A61P009/08 A61P017/06 A61P019/02 A61P029/00 A61P035/00 A61P037/02 A61P043/00 C07D209/12 C07D209/34 C07D231/54 C07D235/02 C07D237/26 C07D239/70 C07D241/36 C07D249/16 C07D251/16 C07D251/72 C07D253/065 C07D253/08 C07D257/04 C07D257/08 C07D257/10 C07D261/08 C07D261/20 C07D263/32 C07D263/52 C07D265/12 C07D265/16 C07D265/34 C07D265/36 C07D271/04 C07D271/06 C07D271/08 C07D271/10 C07D271/12 C07D273/04 C07D275/02 C07D275/04 C07D279/06 C07D279/08 C07D279/12 C07D279/14 C07D279/16 C07D285/06 C07D285/08 C07D285/10 C07D285/12 C07D285/14 C07D285/18 C07D285/20 C07D285/22 C07D285/34 C07D291/02 C07D291/08 C07D307/36 C07D307/38 C07D307/40 C07D307/52 C07D307/77 C07D311/02 C07D311/94 C07D317/04 C07D317/06 C07D401/02 C07D401/32 C07D403/04 C07D471/02 C07D487/02.

☐ 54. WO 9921859A. New azaoxindole derivatives useful for treating diseases characterized by a cellular proliferation. CHEUNG, M, et al. A61K031/435 A61K031/517 C07D000/00 C07D471/04 C07D209:00 C07D221:00 C07D471/04.

☐ 55. US 6268391B. New benzylidene-di:hydro-indolone derivatives - useful as tyrosine kinase inhibitors, e.g. for treating cancer, rheumatoid arthritis or diabetes mellitus. DICKERSON, S, et al. A61K031/40 A61K031/404 A61K031/407 A61K031/41 A61K031/422 A61K031/4245 A61K031/427 A61K031/429 A61K031/437 A61K031/4439 A61K031/496 A61K031/53 A61K031/5377 A61P001/16 A61P003/10 A61P007/02 A61P009/00 A61P009/10 A61P013/12 A61P017/02 A61P017/06 A61P019/02 A61P025/00 A61P029/00 A61P035/00 A61P037/06 A61P043/00 C07D000/00 C07D209/34 C07D209:00 C07D221:00 C07D401/04 C07D401/06 C07D401/12 C07D403/04 C07D403/06 C07D405/06 C07D409/04 C07D409/06 C07D413/04 C07D413/06 C07D417/04 C07D417/06 C07D417/10 C07D417/12 C07D471/04 C07D487/04 C07D513/04 C07D221:00 C07D471/04 C07D235:00 C07D471/04 C07D209:00 C07D221:00 C07D471/04 C07D221:00

Search Results - Record(s) 1 through 2 of 2 returned.

☐ 1. RD 371034A. Screening for inhibitors or ras-protein partner interactions - using a ras-binding domain coated on scintillation plates and tritium-labelled GTP. C12N000/00.

☐ 2. RD 371034A. Screening for inhibitors or ras-protein partner interactions - using a ras-binding domain coated on scintillation plates and tritium-labelled GTP. C12N000/00.

Generate Collection Print

Term	Documents
PARTNER	52969
PARTNERS	35600
(7 AND PARTNER).PGPB,USPT,USOC,EPAB,JPAB,DWPI,TDBD.	2
(L7 AND PARTNER).PGPB,USPT,USOC,EPAB,JPAB,DWPI,TDBD.	2

Prev Page Next Page Go to Doc#

Previous Doc Next Doc Go to Doc#
First Hit

Generate Collection

L6: Entry 6 of 9 File: PGPB Jul 15, 2004

DOCUMENT-IDENTIFIER: US 20040136978 A1
TITLE: Abin-mediated hepatitis protection

Summary of Invention Paragraph:
[0011] Recently, considerable progress has been made in understanding the details of signalling pathways that regulate and mediate NF-.kappa.B activation in response to TNF and IL-1. These cytokines act by binding to specific cell surface receptors, which in turn initiate the recruitment of a number of specific adaptor proteins, and the activation of a kinase complex that phosphorylates the NF-.kappa.B inhibitor I.kappa.B. The latter retains NF-.kappa.B in the cytoplasm in an inactive dimeric form. Once phosphorylated, I.kappa.B is marked for ubiquitination and subsequent degradation by the proteasome, allowing the nuclear translocation of NF-.kappa.B. Whereas members of the I.kappa.B family have been well studied as direct inhibitors of NF-.kappa.B, a number of other proteins have been reported to negatively regulate NF-.kappa.B-dependent gene expression. We and others have previously shown that the zinc finger protein A20 is a potent inhibitor of NF-.kappa.B activation in response to TNF, IL-1, LPS and CD-40 (reviewed in Beyaert et al., 2000). In addition, A20 also exerts an anti-apoptotic function in a number of cell lines. A20 is only expressed upon NF-.kappa.B activation, and is involved in the negative feedback regulation of NF-.kappa.B activation. A20-deficient mice were recently shown to be defective in the termination of NF-.kappa.B activation, leading to strong inflammatory responses and cachexia (Lee et al., 2000). The underlying mechanisms responsible for the inhibition of NF-.kappa.B-dependent gene expression by A20 is still unclear. A20 interacts with the I.kappa.B kinase complex, as well as with TRAF2 and TRAF6, which are part of the I.kappa.B kinase activation cascade initiated by TNF and IL-1/LPS, respectively. In addition, three novel A20-binding proteins (ABIN, ABIN-2 and ABIN-3) were recently isolated. Upon overexpression in cell lines, these proteins were shown to inhibit NF-.kappa.B-dependent gene expression in response to TNF or IL-1 (Beyaert et al., 2000; Heyninck et al., 1999; Van Huffel et al., 2001, Van Huffel et al., unpublished; AJ320534).

Previous Doc Next Doc Go to Doc#

Previous Doc Next Doc Go to Doc#
First Hit

Generate Collection

L6: Entry 7 of 9 File: PGPB Jun 10, 2004

DOCUMENT-IDENTIFIER: US 20040110822 A1
TITLE: Anhydride modified cantharidin analogues useful in the treatment of cancer

Summary of Invention Paragraph:
[0004] Following DNA damage induced by chemotherapy or radiation treatment these checkpoints are responsible for halting cell cycle progression in G.sub.1, S and/or G.sub.2 phases (O'Connor, 1996). The cell undergoes a cell cycle arrest so that the damaged DNA can be repaired before entry into S phase or mitosis. The phase at which the cell cycle is halted will depend upon the type of DNA damaging agent used and the point during the cell cycle that the damage was incurred (O'Connor, 1997). The cell cycle is controlled and regulated by an intricate phosphorylation network (Stein et al., 1998). More particularly, activation of cdk/cyclin complexes requires the phosphorylation of a conserved threonine residue, which are catalysed by CAK kinase, as well as the removal of inhibitory phosphorylations by the phosphatase cdc25. Cdc25 is only active in its phosphorylated form. Therefore, protein phosphatase 2A (PP2A) can inhibit the activation of cdk/cyclin complexes by inhibiting CAK activity and by dephosphorylating cdc25. The G.sub.1/S checkpoint is predominantly regulated by the cdk/cyclin D/E complex that mediates its effects by phosphorylating and inactivating the tumour suppressor protein retinoblastoma (pRb). The phosphorylation of pRb prevents it from interacting with the S-phase transcription factor E2F. E2F controls the transcription of proteins needed for DNA synthesis and entry into S-phase including thymidylate synthase. Accordingly, the inactivation of pRb by phosphorylation permits entry into the S-phase and vice versa. However, protein phosphatase 1 (PP1) can dephosphorylate pRb and inhibit the cell cycle (Durfee et al., 1993). Thus, PP1 and PP2A are both negative regulators of the cell cycle. Inhibition of PP1 and PP2A would abrogate these checkpoints and prematurely force cells through the cell cycle.

Previous Doc Next Doc Go to Doc#

First Hit Previous Doc Next Doc Go to Doc#

Generate Collection Print

L6: Entry 1 of 9 File: PGPB Mar 9, 2006

DOCUMENT-IDENTIFIER: US 20060053499 A1
TITLE: An invertebrate animal model with neurodegenerative phenotype for screening and testing substances

Description of Disclosure:
[0041] AMPK is a heterotrimer, consisting of the catalytic a subunit and a b and g subunit which are required for stabilization of the complex and kinase activity. The activity of the complex is regulated by phosphorylation through an upstream kinase and both phosphorylation as well as dephosphorylation are sensitive to AMP (Davies et al., 1995). For all three subunits different isoforms were identified which assemble to specific AMPK complexes with distinguishable tissue distribution in vertebrates (Stapleton et al., 1996; Thornton et al, 1998). Whether these different AMPK isoforms have distinguishable physiological functions is still unclear. In contrast to vertebrates where various isoforms are encoded by separate genes, the Drosophila g subunits are created by alternative splicing because we could not identify additional genes in the Drosophila Genome Project. Surprisingly, the Drosophila loe proteins contain domains not described in other species so far. That these domains play a pivotal role for the specific function of the different splice forms is shown by the rescue experiments using different splice forms and deletion constructs which do not or only partially restore the wild type function. In addition, the failing rescue experiments suggest that the function of the various isoforms goes beyond the regulation of the energy demand and very likely the same applies to vertebrate isoforms.

Previous Doc Next Doc Go to Doc#

L6: Entry 2 of 9 File: PGPB Feb 9, 2006

DOCUMENT-IDENTIFIER: US 20060030616 A1
TITLE: Anhydride modified cantharidin analogues useful in the treatment of cancer

Brief Summary Text:
[0003] Following DNA damage induced by chemotherapy or radiation treatment these checkpoints are responsible for halting cell cycle progression in G.sub.1. S and/or G.sub.2 phases (O'Connor, 1996). The cell undergoes a cell cycle arrest so that the damaged DNA can be repaired before entry into S phase or mitosis. The phase at which the cell cycle is halted will depend upon the type of DNA damaging agent used and the point during the cell cycle that the damage was incurred (O'Connor, 1997). The cell cycle is controlled and regulated by an intricate phosphorylation network (Stein et al., 1998). More particularly, activation of cdk/cyclin complexes requires the phosphorylation of a conserved threonine residue, which are catalysed by CAK kinase, as well as the removal of inhibitory phosphorylations by the phosphatase cdc25. Cdc25 is only active in its phosphorylated form. Therefore, protein phosphatase 2A (PP2A) can inhibit the activation of cdk/cyclin complexes by inhibiting CAK activity and by dephosphorylating cdc25. The G.sub.1/S checkpoint is predominantly regulated by the cdk/cyclin D/E complex that mediates its effects by phosphorylating and inactivating the tumour suppressor protein retinoblastoma (pRb). The phosphorylation of pRb prevents it from interacting with the S-phase transcription factor E2F. E2F controls the transcription of proteins needed for DNA synthesis and entry into S-phase including thymidylate synthase. Accordingly, the inactivation of pRb by phosphorylation permits entry into the S-phase and vice versa. However, protein phosphatase 1 (PP1) can dephosphorylate pRb and inhibit the cell cycle (Durfee et al., 1993). Thus, PP1 and PP2A are both negative regulators of the cell cycle. Inhibition of PP1 and PP2A would abrogate these checkpoints and prematurely force cells through the cell cycle.

L6: Entry 3 of 9 File: PGPB Oct 27, 2005

DOCUMENT-IDENTIFIER: US 20050239134 A1

TITLE: Combinatorial libraries of phosphorothioate single-stranded DNA aptamers for TGF-beta-1 protein

Summary of Invention Paragraph:

[0030] The cognate TGF-.beta. ligand initiates signaling by binding to and bringing together type I and type II receptor serine/threonine kinases on the cell surface, thus forming an "active receptor signaling complex," which allows the type II receptor to phosphorylate the type I receptor kinase domain and which propagates the signal by directly phosphorylating the "receptor-regulated" R-Smad proteins (Smads 2, 3 involved in TGF-.beta. subfamily signaling and Smads 1, 5 and 8 involved in BMP subfamily signaling). Once activated, the R-Smads undergo homotrimerization and form a heteromeric complex with the Co-Smad (Smad 4). The R-Smad-Co-Smad complex translocates to the nucleus of the cell, and in conjunction with other nuclear factors, regulates the transcription of genes. The inhibitory I-Smads (Smads 6, 7) may regulate negatively TGF-.beta. signaling by competing with R-Smads for either receptor or Co-Smad interaction and/or by targeting the receptors for ubiquitination and degradation. The aptamers and thioaptamers of the present invention may be used alone or in combination, e.g., by providing two or more thioaptamers that are specific for the TGF-.beta. ligand and/or its receptors and/or inhibitors to provide combination thioaptamer therapy.

Previous Doc Next Doc Go to Doc#

Detail Description Paragraph:

[0191] Next, it was determined whether Cln/Cdc28, present in small amounts in the ubiquitination reaction, is also required for additional steps in the ubiquitination process (e.g, to phosphorylate the ubiquitination machinery). This was accomplished by treating bacterial Sic1 with Cln2/Gst-Cdc28 complexes immobilized on GSH-Sepharose beads, removing the complexes from the beads prior to use in ubiquitination reactions, and determining whether the complexes were free of soluble kinase by immunoblotting with anti-HA antibodies (See, FIG. 3D, lane 3). These results indicated that Sic1 phosphorylated in this manner was also efficiently ubiquitinated (See, FIG. 3D, lane 9). Thus, these data indicated that Sic1 phosphorylation constitutes the primary requirement of Cln/Cdc28 kinases in Sic1 ubiquitination in the in vitro reaction.

Previous Doc Next Doc Go to Doc#

US 20040166530A1

(12) Patent Application Publication (10) Pub. No.: US 2004/0166530 A1

Harper et al. (43) Pub. Date: Aug. 26, 2004

(54) F-BOX PROTEINS AND GENES

(76) Inventors: Jeffrey Wade Harper, Sugarland, TX (US); Stephen J. Elledge, Houston, TX (US); Jeffrey T. Winston, Thousand Oaks, CA (US)

Correspondence Address:
Tim Headley
GARDERE WYNNE SEWELL LLP
1000 Louisiana, Suite 3400
Houston, TX 77002-5007 (US)

(21) Appl. No.: 09/801,348

(22) Filed: Mar. 7, 2001

Related U.S. Application Data

(60) Division of application No. 09/172,841, filed on Oct. 15, 1998, now Pat. No. 6,232,081.

Continuation-in-part of application No. 08/951,621, filed on Oct. 16, 1997, now Pat. No. 6,573,094.

Publication Classification

(51) Int. Cl.[7] G01N 33/53

(52) U.S. Cl. 435/7.1

(57) ABSTRACT

The present invention provides compositions and methods for gene identification, as well as drug discovery and assessment. In particular, the present invention provides components of an E3 complex involved in ubiquitination of cell cycle regulators and other proteins, as well as members of a class of proteins that directly function in recognition of ubiquitination targets. The present invention also provides sequences of multiple F-box proteins.

Error: **Unable to store job at printer**

Reason: **Printer not configured to collate**

Solution: **Install an EIO hard disk.**

What is claimed is:

1. A method for the detection of one or more NF-κB regulatory factors comprising the steps of:

a) providing a slimb protein, and a sample suspected of containing one or more NF-κB regulatory factors; and

b) exposing said slimb protein to said sample under conditions such that said slimb protein binds to said one or more NF-κB regulatory factors to form a slimb/regulatory factor complex.

2. The method of claim 1, further comprising the step of detecting said slimb/regulatory factor complex.

3. The method of claim 1, further comprising the step of observing said slimb/regulatory factor complex for degradation of said one or more NF-κB regulatory factors.

4. The method of claim 1, further comprising the step of exposing said slimb protein and one or more NF-κB regulatory factors to an F-box protein antagonist.

5. The method of claim 4, wherein said F-box protein antagonist prevents the formation of said slimb/regulatory factor complex.

6. A method for the detection of a slimb protein complex, comprising the steps of:

a) providing a slimb protein and a sample suspected of containing one or more proteins capable of forming a complex with said slimb protein; and

b) exposing said slimb protein to said one or more proteins capable of forming a complex with said slimb protein under conditions such that said slimb protein binds to said one or more proteins capable of forming a complex with said slimb protein to form a slimb protein complex.

7. The method of claim 6, further comprising the step of detecting said slimb protein complex.

8. The method of claim 6, wherein step b) further comprises exposing said slimb protein and said one or more proteins capable of forming a complex with said slimb protein to an F-box protein antagonist.

9. The method of claim 8, wherein said F-box protein antagonist prevents the formation of said slimb protein complex.

10. An isolated nucleotide sequence comprising nucleotide sequence encoding at least one functionally active fragement of an F-box protein, wherein said sequence consists of a least a portion of the sequence set forth in SEQ ID NOS: 54 and 56.

* * * * *

L6: Entry 4 of 9 File: PGPB Apr 7, 2005

DOCUMENT-IDENTIFIER: US 20050074834 A1

TITLE: Method and system for classifying a scenario

Detail Description Paragraph:

[0351] Adenylate cyclase and other cell surface effector proteins mediate signals between upstream receptor binding events and downstream regulatory cues involved in the digitization of incoming analog signals. V. J. Watts reviews the molecular mechanisms of adenylate cyclase activation. The nine-membrane bound isoforms of the enzyme adenylate cyclase (AC) are highly regulated by neurotransmitters and drugs acting through GPCRs to modulate intracellular cAMP levels (V. J. Watts, Molecular Mechanisms for Heterologous Sensitization of Adenylate Cyclase, J. Pharm. Exptl. Thera. 302(1), 1-7, 2002). G. G. Kelley and others report on the mechanisms and regulation of phospholipase-C.epsilon., a recently discovered effector molecule with many activities distinct from those of other phospholipase isoforms, particularly with respect to the manner of involvement of GPCRs in its regulatory cascade (G. G. Kelley, S. E. Reks and A. V. Smrcka, Hormonal regulation of phospholipase C.epsilon. through distinct and overlapping pathways involving G12 and Ras family G-proteins, Biochem. J., 378, 129-139). Heterologous and homologous phosphorylation and dephosphorylation of effector and receptor complexes by PKA and receptor-specific kinases and other regulatory molecules result occurs in response in ligand concentration. High concentrations of ligand result in down regulation of receptors with high specifities for the binding ligand through phosphorylation by receptor specific kinases. Lower concentrations of ligand result in a rhythmic phosphorylation/dephosporylation of affected receptors digitizing incoming signals.

Previous Doc Next Doc Go to Doc#

Oncogene (2000) 19, 6594–6599

www.nature.com/onc

Development of anticancer drugs targeting the MAP kinase pathway

Judith S Sebolt-Leopold*,[1]

[1]*Pfizer Global Research and Development, Ann Arbor Laboratories, Ann Arbor, Michigan, MI 48105, USA*

Since the discovery of the role of ras oncogenes in tumorigenesis, we have witnessed an explosion of research in the signal transduction area. In the quest to understand how Ras transmits extracellular growth signals, the MAP kinase (MAPK) pathway has emerged as the crucial route between membrane-bound Ras and the nucleus. The MAPK pathway encompasses a cascade of phosphorylation events involving three key kinases, namely Raf, MEK (MAP kinase kinase) and ERK (MAP kinase). This kinase cascade presents novel opportunities for the development of new cancer therapies designed to be less toxic than conventional chemotherapeutic drugs. Furthermore, as a signal transduction-based approach to cancer treatment, inhibition of any one of these targets has the potential for translational pharmacodynamic evaluation of target suppression. The rationale for targeting the MAP kinase pathway will be reviewed here along with a discussion of various pharmacological approaches and the promise they hold for a new generation of anticancer drugs. *Oncogene* (2000) **19**, 6594–6599.

Keywords: mitogen-activated protein kinase (MAPK); extracellular signal-regulated kinase (ERK); MAP kinase kinase (MEK); raf

Introduction

Many receptor tyrosine kinases and cytokine receptors in association with heterotrimeric G proteins are known to activate intracellular protein serine/threonine kinases termed mitogen-activated protein kinases (MAPKs). Of the various families of MAPKs, which are also referred to as extracellular signal-regulated kinases (ERKs), the first to be characterized were ERK1 and ERK2. Both of these ERKs are activated in response to diverse extracellular stimuli and by protooncogene-encoded proteins that induce proliferation. A cascade of phosphorylation events downstream from Ras activates these kinases. Upstream regulation of the MAP kinase pathway is complex as evidenced by the number of functions fulfilled by its activation. Processes impacted by MAPK activation encompass the cytoplasm, nucleus, cytoskeleton, and the membrane. The reader is referred elsewhere for comprehensive reviews on the subject of regulation through MAPK cascades (Cobb, 1999; Lewis *et al*, 1998; Kolch, 2000).

The Raf-MEK-ERK pathway represents one of the best characterized Ras signaling pathways. Raf and MEK have consequently emerged as key protein kinases to target for anticancer drug design. While there exist multiple MAP kinase families, e.g. jun kinase and p38, which are also activated downstream of low molecular weight G-proteins, ERK has been the best characterized and is more pertinent to aberrant signaling in human cancer. For some cancers, especially those of hematopoietic origin, the p38 and jun kinase pathways may in fact yield targets exploitable for anticancer drug development. However, a broad array of solid tumors is known to express constitutive levels of phosphorylated ERK1 and ERK2. Activation of ERK is critical for a large number of Ras-induced cellular responses. Included among these responses is transcriptional activation of multiple genes (Hill and Treisman, 1995). The best-characterized physiological substrates of ERK are ternary complex factors (TCFs), which are directly phosphorylated by ERK to activate their transcription activation potential (Gille *et al.*, 1992; Janknect *et al.*, 1993; Marais *et al.*, 1993). TCFs, in association with serum response factor, is thought to be critical for the activation of numerous mitogen-inducible genes (Hill and Treisman, 1995).

Many molecules ultimately contribute to activation of the Ras-ERK pathway, including a number that are involved in protein-protein interactions. With respect to pharmacological intervention, it is generally difficult to selectively target the binding site shared by two proteins. It is therefore not coincidental that the development of agents targeting the Ras-MAPK pathway has largely focused on the design of small molecule inhibitors of enzyme function. As will be explored in more detail below, four proteins have emerged as key players in the quest to intervene in this pathway: Ras, Raf, MEK (MAP kinase kinase), and ERK. Ras is the subject of a paper that appears elsewhere in this review issue and therefore will not be covered further here.

Rationale for targeting the MAP kinase pathway

Figure 1 provides a simplified schematic representation of the signaling events leading to activation of the MAP kinase pathway. Initially, Ras interacts with and activates the serine/threonine protein kinase Raf1 in a GTP-dependent manner (Daum *et al.*, 1994; Stokoe *et al.*, 1994). A family of Raf protein kinases has been identified and is comprised of A-Raf, B-Raf, and c-Raf1. It has been suggested that this family of kinases, which is known to regulate proliferation, differentiation, and apoptosis, have both overlapping and unique regulatory functions (Hagemann and Rapp, 1999). For example, transfection of oncogenic H-ras led to a preferential activation of endogenous c-Raf1 as opposed to A-Raf (Weber *et al.*, 2000). Mutated Raf-1 is constitutively active and possesses *in vitro* transforming potential (Stanton and Cooper, 1987). The potential for Raf-1 to play a broad role in tumorigenesis is evidenced by its ability to become activated by either PKCα or the antiapoptotic protein Bcl-2 in a Ras-independent manner (Kolch *et al.*, 1993; Wang *et al.*, 1996).

*Correspondence: JS Sebolt-Leopold

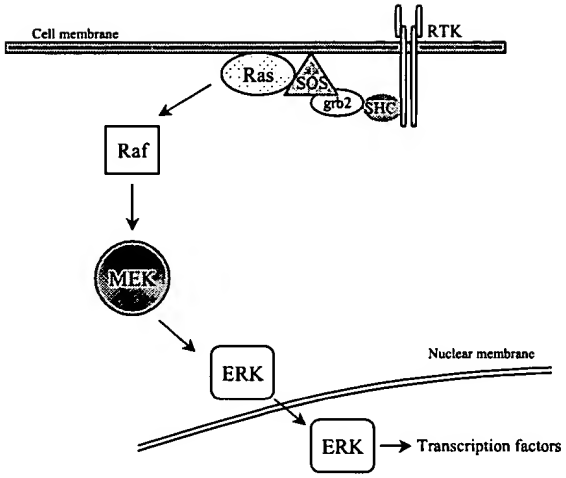

Figure 1 Schematic representation of the Ras–MAP kinase pathway. The MAP kinase cascade contains three sequential kinases: a MAP kinase kinase kinase (Raf), MAP kinase kinase (MEK), and MAP kinase (ERK)

Importantly, *raf* mutations have been identified in a range of human tumors (Storm and Rapp, 1993). Independent of its mutation status, Raf is also activated in tumor cells containing enhanced growth factor signaling pathways, such as those induced by mutant or constitutively expressed Ras or EGF receptor family members. Therefore, the collective evidence suggests that Raf-1 is a viable anticancer drug target.

Alternatively, targeting the molecule immediately downstream from Raf, that is, the dual specificity kinase MEK can also be envisioned as representing a rational approach to anticancer drug design. Subsequent to its activation, Raf-1 phosphorylates and activates both MEK1 and MEK2 (hereafter referred to as MEK) on two distinct serine residues (Dent *et al.*, 1992; Crews *et al.*, 1992; Her *et al.*, 1993). Activated MEK then phosphorylates ERK1 and ERK2 on both a tyrosine and a threonine residue (Anderson *et al.*, 1990). No substrates for MEK have been identified other than ERK1 and ERK2 (Seger *et al.*, 1992). This tight selectivity in addition to the unique ability to phosphorylate both tyrosine and threonine residues are consistent with this kinase playing a central role in integration of signals into the MAPK pathway. Constitutive activation of MEK has been shown to result in cellular transformation (Cowley *et al.*, 1994; Mansour *et al.*, 1994). While MEK has not been identified as an oncogene product, MEK is the focal point of many signal transduction mitogenic pathways activated by proven oncogenes. Pivotal studies carried out with the MEK inhibitor PD98059 provided further impetus for exploring whether MEK could be exploited as a target for rational anticancer drug design. In these studies, MEK inhibition not only impaired proliferation, but also impacted a diverse array of cellular events, including differentiation, apoptosis, and angiogenesis (Dudley *et al.*, 1995; Alessi *et al.*, 1995; Pages *et al.*, 1993; Pang *et al.*, 1995; Finlay *et al.*, 2000; Holmstrom *et al.*, 1999; Elliceiri *et al.*, 1998; Milanini *et al.*, 1998). Based on these collective findings, MEK therefore represents an attractive target for pharmacological intervention in cancer.

Theoretically, it could be argued that intervention in any of the kinase events in the MAPK cascade could represent a viable approach to crippling tumor growth. If so, then Raf-1, MEK, and ERK all emerge as reasonable anticancer drug targets. The advent of high volume screening of pharmaceutical libraries for small molecule inhibitors has most certainly produced reasonable drug candidates targeting all steps of this pathway. For example, a cascade assay has been reported that is capable of identifying inhibitors of cRaf1, MEK1, or ERK2 (McDonald *et al*, 1999). As we now turn to preclinical and clinical evaluation of these small molecule inhibitors, it is important to keep in mind that their ultimate promise or differences may depend as much on their pharmacological attributes as on the merits of their targeted kinase.

It should be noted that the identification of pathway components in the Ras-MAP kinase pathway is likely incomplete. For example, a Raf-1-interacting protein, RKIP, has recently been reported (Yeung *et al.*, 1999). This protein inhibits the phosphorylation and activation of MEK by Raf-1 and has also been shown to co-localize with Raf-1. It has been proposed that RKIP binding to either Raf-1 or MEK dissociates Raf-MEK complexes, thereby interrupting MEK activation and downstream signaling (Yeung *et al.*, 2000). Discovered with the use of a yeast two-hybrid system, the relevance of RKIP expression to signal transduction in tumor cells is unclear at the present time. Furthermore, until we learn whether RKIP expression is negatively regulated, it remains unclear how to pharmacologically elevate its expression to impair tumor growth. Although highly speculative based on our current knowledge of the role of RKIP, it is conceivable that elevated expression of this protein could offer tumor cells a mechanism of resistance to MAPK pathway inhibitors.

There exist a multitude of other newly discovered proteins that may provide insight into the design of novel signal transduction-based cancer therapies that exploit the MAP kinase pathway. These include Sur-8, which is thought to act as a scaffold to enhance Ras-MAP kinase signaling by facilitating Ras-Raf interaction (Li *et al.*, 2000), as well as the kinase suppressor of Ras (KSR). KSR is also thought to act as a scaffolding protein for the Ras-MAPK pathway (Stewart *et al.*, 1999). Another interesting protein is MP-1, which has been reported to enhance activation of the MAPK by binding MEK (Schaeffer *et al.*, 1998). Last but not least, a novel ERK has recently been identified, ERK1b, which is an alternatively spliced form of ERK1, that appears to be elevated in Ras-transformed cells (Yung *et al.*, 2000).

Amenability of the MAP kinase pathway to pharmacodynamic evaluation

Using an antibody specific for dually phosphorylated ERK1 and ERK2, *in vivo* evaluation of MEK inhibition can easily be measured in excised samples. The utility of such an assay in preclinical animal models was demonstrated for the MEK inhibitor PD184352 (Sebolt-Leopold *et al.*, 1999). Phosphorylated MAPK is the product of MEK activity and thus represents a direct measure of MEK inhibition. Using an antibody specific for phosphorylated MEK, *ex vivo* evaluation for Raf inhibition should likewise be straightforward. However, pharmacodynamic evalua-

tion of ERK inhibitors would be complex, as multiple nuclear proteins and transcription factors are substrates for phosphorylated ERK.

At the preclinical stage, pharmacodynamic assays are not only useful for optimizing the design of dosing regimens, but also offer the advantage of being able to correlate antitumor efficacy with inhibition of the biochemical target. A large number of cell lines as well as primary human tumors have been surveyed for constitutive activation of the MAPK pathway (Hoshino *et al.*, 1999). It will be of interest to correlate the degree of target expression of a given tumor with its inherent sensitivity to agents directed against that target. The data obtained thus far with the MEK inhibitor PD184352 suggest that tumors containing high level expression of phosphorylated MAP kinase are most sensitive to treatment with this agent (Sebolt-Leopold *et al.*, 1999). There are obvious clinical implications if this pattern continues during the expansion of our database; such assays could then be exploited as prognostic tools to identify those patients most likely to derive therapeutic benefit from treatment with a given agent.

Figure 2 demonstrates the applicability of pharmacodyamic evaluation of PD184352 to a range of tissues or cells (Sebolt-Leopold, unpublished data). Twenty-four hours after an oral dose of 200 mg/kg was administered to monkeys, significant inhibition of MAPK phosphorylation was observed in lung as well as skin tissue (Figure 2a,b, respectively). Looking ahead to the clinical setting, biomarker evaluation of phosphorylated MAPK levels can also be measured in PMA-stimulated peripheral blood mononuclear cells (Figure 2c). Such assays have the potential to define a dose threshold that delivers total suppression of the desired target. For a target such as MEK that is thought to offer tumor-specific pharmacologic effects, Phase II trials may not need to be carried out at the MTD determined from Phase I studies.

Pharmacological approaches to targeting the MAPK pathway

The only Raf-directed approach for which preclinical efficacy data have been published is that employing a c-raf-1 antisense oligonucleotide. ISIS 5132 is a 20-base phosphorothioate antisense oligodeoxynucleotide designed to hybridize to 3′ untranslated sequences of c-raf-1 mRNA (Monia *et al.*, 1996). Reduction of c-raf-1 mRNA was shown to occur in tumor-bearing mice treated with relatively low doses. Importantly, preclinical efficacy and toxicology studies suggested a large therapeutic window for ISIS 5132 (Henry *et al.*, 1997). Early clinical data have recently been reported with ISIS 5132 (Stevenson *et al.*, 1999; Yuen and Sikic, 2000). This agent was well tolerated and suppression of target gene expression was observed in peripheral blood mononuclear cells (O'Dwyer *et al*, 1999). However, Phase II data have not yet been published.

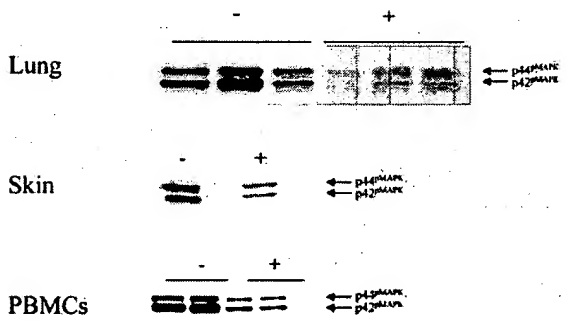

Figure 2 Effects of the MEK inhibitor PD184352 on phosphorylated MAP kinase (pMAPK) levels in (a) monkey lung, (b) monkey skin, and (c) human peripheral blood mononuclear cells (PBMCs). Monkeys were administered an oral dose of 200 mg/mg PD184352 (+) or diluent (−) followed 24 h later by excision of the indicated tissue for analysis of pMAPK. Human whole blood was spiked with 1 μM PD184352 followed immediately by stimulation with PMA and isolation of PBMCs for evaluation of pMAPK levels

It is anticipated that clinical data will soon emerge from testing of small molecule inhibitors of raf kinase. Based on the patent literature, several classes of substituted ureas have been identified as raf kinase inhibitors (Bayer, 1999a,b,c, 2000). Benzamides have also been investigated for their raf kinase inhibitory activity (Zeneca, 1998). It has been reported that a potent and specific inhibitor of Raf isoforms *in vitro*, ZM 336372 paradoxically induces significant activation of c-Raf without inducing any activation of MEK1 or ERK2 (Hall-Jackson *et al.*, 1999). The authors speculate that Raf may suppress its own activation by virtue of a novel feedback loop. If so, then inhibition would be counterbalanced by reactivation which would limit the utility of raf kinase inhibitors as anticancer agents. Clinical testing of raf kinase inhibitors will likely clarify this paradox. It should also be noted that growth factor-stimulated ERK is capable of retrophosphorylating MEK in a negative feedback fashion (Brunet *et al.*, 1994). Yet, MEK inhibitors, e.g. PD184352, clearly exhibit promising preclinical activity in a number of human and murine tumor models. This suggests that the retrophosphorylation-derived negative regulation does not inactivate the pathway.

An orally active small molecule inhibitor of MEK has provided *in vivo* validation for targeting MEK for anticancer drug design (Sebolt-Leopold *et al.*, 1999). In this study, PD184352, a non-ATP-competitive, highly selective inhibitor of MEK, was found to significantly inhibit growth of colon carcinomas of both mouse and human origin. Importantly, efficacy was achieved at well tolerated doses and was correlated with a reduction in the levels of activated MAPK in excised tumors. In addition to impairing tumor proliferation, PD184352 was found to block the disruption of cell-cell contact and motility required for invasion. This finding is consistent with earlier reports indicating that hepatocyte growth factor (HGF) induces dispersion of epithelial cells by a Ras-dependent mechanism. The MEK/MAPK pathway is an essential mediator of HGF-induced cell scattering (Ridley *et al.*, 1995; Herrera, 1998; Potempa and Ridley, 1998; Tanimura *et al.*, 1998). PD184352 (now designated CI-1040) is presently undergoing Phase 1 evaluation in cancer patients.

Once activated, a fraction of cytoplasmic ERK1 and ERK2 translocates into nuclei (Lenormand *et al*, 1993). In this way, these MAP kinases enable the regulation of gene expression by phosphorylation of nuclear transcription factors. While selective ERK1/ERK2 inhibitors have not been described in the literature,

intervention in activation of transcription factors may prove to be an exploitable approach for anticancer drug development. Until ERK inhibitors are evaluated, the pharmaceutical attractiveness of this kinase is left to speculation. It is not clear whether direct inhibition of ERK would prove to be more toxic than inhibition of the upstream kinases Raf and MEK. Whereas a null mutation in the MEK1 gene proved to be embryonic lethal, ERK1 knockout mice were viable and of normal size (Giroux *et al.*, 1999; Pages *et al* 1999). However, in the case of the p38 MAP kinase family, it has been shown that p38 null mutants result in an embryonic lethal phenotype, unlike the case for MKK3 knockouts (Allen *et al.*, 2000; Lu *et al.*, 1999). Aside from unresolved theoretical concerns regarding potential toxicities, *a priori* ERK and MEK inhibitors might be expected to act similarly since ERK can only be activated by MEK. This is in contrast to the situation in the p38 pathway where p38 can be activated by three distinct MKKs. Therefore, to target the pertinent upstream MAP kinase kinases in the p38 pathway, it might prove necessary to abolish activity of not one but three enzymes, namely MKK3, MKK4, and MKK6.

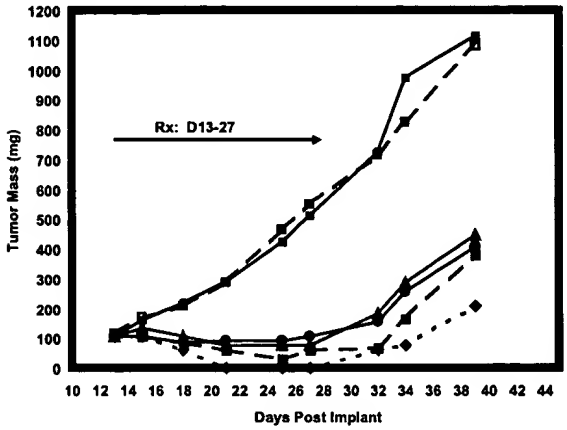

Figure 3 Effects of oral PD184352 (CI-1040) treatment on growth of staged pancreatic BxPc3 xenografts. Treatments of mice bearing subcutaneous implants of BxPc3 tumors was initiated when tumors reached 100 mg in size. PD18452 was administered orally three times a day on days 13 through 26 post-implantation. Doses administered were 200 mg/kg (◆), 124 mg/kg (■, dashed line), 77 mg/kg (●), and 48 mg/kg (▲). Controls plotted here included untreated animals (□) and diluent-treated animals (■, solid line)

Blockade of the MAP kinase pathway exerts pleiotropic effects exploitable in future clinical trial design

Of the numerous therapeutic approaches to cancer treatment, most take the form of a single-pronged attack aimed at either: (1) slowing of tumor growth, (2) inhibition of invasion and metastasis, (3) induction of tumor cell death, or (4) promotion of tumor differentiation. However, by pharmacological intervention of the MAP kinase pathway, one can envision a single agent that concurrently exploits more than one of these processes. Perhaps the best evidence in support of this statement is provided by the collective data obtained with MEK inhibitors.

Greater than 1500 references appear in the literature describing the utility of PD98059 in elucidating the role of the MAP kinase pathway in diverse cellular processes. The involvement of this pathway in tumor proliferation is well documented. While not all tumors rely on MAP kinase activation to drive their growth, a significant percentage of human tumors do in fact exhibit constitutive activation of the MAPK pathway (Hoshino *et al.*, 1999). MEK inhibition has been shown to effectively shut down tumor growth *in vivo* in a cytostatic manner (Sebolt-Leopold *et al.*, 1999). However, MEK inhibition has also proven to induce tumor regressions in some xenograft models, e.g. pancreatic BxPc3, as exemplified in Figure 3 (Merriman and Sebolt-Leopold, unpublished data). These results are consistent with an increase in apoptosis occurring in response to MEK inhibition. This is perhaps not surprising in view of evidence that one of the phosphorylation sites on the pro-apoptotic molecule BAD, i.e. serine-112, is phosphorylated by MAP kinase (Fang *et al.*, 1999; Scheid *et al.*, 1999). Phosphorylation of this site results in loss of the ability of BAD to heterodimerize with the survival protein BCL-2. Thus, by promoting interaction between BAD and BCL-2, it appears feasible that inhibition of MEK would serve to increase the incidence of apoptosis. In BxPc3 tumors that had regressed in response to treatment with the MEK inhibitor CI-1040, reduced phosphorylation of the serine-112 site on BAD was demonstrated *ex vivo* (Sebolt-Leopold, unpublished results). It should also be noted that a recent report indicates that activation of the MAPK pathway acts to protect pancreatic tumor cells from apoptosis by regulating expression of Bcl-2 (Boucher *et al.*, 2000).

Inhibition of MAP kinase signaling is also anticipated to result in anti-metastatic and anti-angiogenic effects. Activation of the MAPK pathway occurs in response to integrin-mediated cellular adhesion to the extracellular matrix, which plays a critical role in both tumor metastasis and angiogenesis (Chen *et al.*, 1994; Zhu and Assoian, 1995). It was recently reported that active ERK is targeted to newly formed focal adhesions after integrin engagement of v-Src activation, providing support for a role for ERK in regulation of adhesion (Fincham *et al.*, 2000). Transfection of constitutively active MEK, which resulted in increased expression of matrix metalloproteinases 2 and 9 as well as cathepsin L, resulted in macroscopic metastases (Welch *et al.*, 2000). It is therefore not surprising that MEK inhibition in colon tumor models resulted in decreased invasiveness as well as inhibition of cell motility (Sebolt-Leopold *et al.*, 1999). It is also anticipated that inhibition of MAPK signaling will negatively impact angiogenesis. Such an effect is likely based on our knowledge of sustained activation of MAPK being required for angiogenesis (Eliceiri *et al.*, 1998). MAPK activation is probably also required for growth factor-induced secretion of angiogenic growth factors from tumor cells (Petit *et al.*, 1997).

Therefore, evidence would seem to suggest that single agent treatment with a drug targeted against the MAPK pathway could potentially impair tumor survival by more than one of the therapeutic approaches outlined above. It is likely however that the design of future clinical trials with MAPK pathway

inhibitors will attempt to boost therapeutic kill by employing combination regimens. Two classes of chemotherapeutic agents of particular interest in this regard are mitotic inhibitors, e.g. taxanes, as well as platinum-coordination complexes, e.g. cisplatin and carboplatin. The kinetochore motor protein CENP-E, which was found *in vivo* to associate preferentially with active MAPK during mitosis, was also phosphorylated by MAPK at sites known to regulate its interactions with microtubules (Zecevic *et al.*, 1998). These investigators propose that MAP kinase may play a role in mitosis by affecting the ability of CENP-E to mediate interactions between microtubules and chromosomes. Cell culture experiments have shown that the combination of taxol with the MEK inhibitor CI-1040 results in a significant increase in apoptotic frequency that is greater than that predicted from the additive effects of each agent tested alone (Sebolt-Leopold, unpublished data).

With respect to platinum coordination complexes, cisplatin treatment of ovarian carcinoma cells or HeLa cells has been reported to result in induction of ERK activity (Persons *et al.*, 1999; Wang *et al.*, 2000). Furthermore, inhibition of cisplatin-induced ERK activity by the MEK inhibitor PD98059 resulted in enhanced cytotoxicity in response to cisplatin treatment. Thus the combination of cisplatin or carboplatin with MAPK pathway inhibitors warrants further investigation for potential clinical benefit. The p53 phenotype may play a role in determining whether the combination of a MAPK signaling antagonist with a cytotoxic agent results in synergistic cell kill, since a link has been established between p53 signaling and the MAPK cascade. It has been reported that treatment of normal cells with DNA-damaging agents induced ERK activation in a p53-dependent manner, whereas tumor-derived p53 mutants that were defective in DNA-binding failed to activate ERK (Lee *et al.*, 2000). Interestingly, it was recently reported that inhibition of ERK activation by MEK inhibition resulted in decreased accumulation of p53 during exposure to cisplatin (Persons *et al.*, 2000). These investigators further showed that p53 was phosphorylated by ERK *in vitro* in an event antagonized by MEK inhibition during cisplatin treatment. Thus it appears likely that ERK activation induced by cisplatin regulates the p53 response to cytotoxic damage induced by this DNA-damaging agent.

While only two examples have been given here, one could rationalize the combination of a MAPK pathway inhibitor with a multitude of other agents. If single agent treatment with MAPK signaling antagonists proves to be well tolerated upon chronic dosing, it is tempting to speculate that these drugs might also prove useful in preventing the emergence of hormone-resistant cancers. For example, estrogen-dependent breast cancers that initially respond to tamoxifen treatment frequently become resistant. It has been shown that this shift in hormone-response pattern is accompanied by a shift from MAPK-independent to MAPK-dependent cell growth (Lange *et al.*, 1996). Along these same lines, data exist in support of increased activation of the MAP kinase pathway as prostate cancer progresses to a more advanced and androgen-independent state (Gioeli *et al.*, 1999).

Looking to the future

The ultimate therapeutic promise of signaling antagonists directed against the MAPK pathway can only be determined from human testing. Until we have gained clinical experience on their safety and efficacy profiles, arguing the merits of targeting one kinase versus another remains an academic exercise. Clearly, the chances for clinical success will be enhanced if human trials are designed to exploit the mechanism of action of the agent under study. Combination regimens employing chemotherapeutic agents have generally been driven by safety considerations, i.e. combination of cytotoxic agents with non-overlapping toxicities. With the development of signaling antagonists that are considerably less toxic, it will be important to turn our attention to combining MAPK pathway inhibitors with cytotoxic agents or with other signaling antagonists based on anticipated mechanistic-based synergy.

As these agents enter the clinic, reagents will be available to directly monitor target suppression. The use of such biomarker analysis will not only aid dose escalation, but also offers the advantage of correlating efficacy with the degree of activity anticipated by the extent of target suppression. The advantages of having pharmacodynamic assays available for analysis of clinical samples can not be overstated. The field of clinical oncology is plagued by examples of negative clinical trials, where it is not clear if lack of efficacy was due to the inhibited target being inconsequential to outcome or simply whether the requisite degree of target inhibition was not achieved. Furthermore, if retrospective data analysis from clinical trials shows that the degree of target expression correlates well with sensitivity to the test agent, then this has obvious prognostic implications when tumor biopsy material is available.

In summary, the next decade will no doubt represent a very exciting time in the field of clinical oncology, as a number of signaling antagonists, including inhibitors of the MAP kinase pathway, get put to the real test.

Acknowledgments

The author would like to thank Drs Roman Herrera, Jennifer Swanteck and WR Leopold for helpful comments in their critical review of this manuscript.

References

Alessi DR, Cuenda A, Cohen P, Dudley DT and Saltiel AR. (1995). *J. Biol. Chem.*, **270**, 27489–27494.

Allen M, Svensson L, Roach M, Hambor J, McNeish J and Gabel CA. (2000). *J. Exp. Med.*, **191**, 859–869.

Anderson NG, Maller JL, Tonks NK and Sturgill TW. (1990). *Nature*, **343**, 651–653.

Bayer Corporation. (1999a). WO9932106.

Bayer Corporation. (1999b). WO9932436.

Bayer Corporation. (1999c). WO9932455.

Bayer Corporation. (2000). WO200042012.

Boucher MJ, Morisset J, Vachon PH, Reed JC, Laine J and Rivard N. (2000). *J. Cell. Biochem.*, **79**, 355–369.

Brunet A, Pages G and Pouyssegur J. (1994). *FEBS Lett.*, **346**, 299–303.

Chen Q, Kinch MS, Lin TH, Burridge K and Juliano RL. (1994). *J. Biol. Chem.*, **269**, 26602–26605.
Cobb MH. (1999). *Prog. Biophys. Mol. Biol.*, **71**, 479–500.
Cowley S, Paterson H, Kemp P and Marshall CJ. (1994). *Cell*, **77**, 841–852.
Crews CM, Alessandrini A and Erikson RL. (1992). *Science*, **258**, 478–480.
Daum G, Eisenmann-Tappe I, Fries HW, Troppmair J and Rapp UR. (1994). *Trends Biochem. Sci.*, **19**, 474–480.
Dent P, Haser W, Haystead TA, Vincent LA, Roberts TM and Sturgill TW. (1992). *Science*, **257**, 1404–1407.
Dudley DT, Pang L, Decker SJ, Bridges AJ and Saltiel AR. (1995). *Proc. Natl. Acad. Sci. USA*, **92**, 7686–7689.
Eliceiri BP, Klemke R, Stromblad S and Cheresh DA. (1998). *J. Cell Biol.*, **141**, 1255–1263.
Fang X, Yu S, Eder A, Mao M, Bast Jr RC, Boyd D and Mills GB. (1999). *Oncogene*, **18**, 6635–6640.
Fincham VJ, James M, Frame MC and Winder SJ. (2000). *EMBO J.*, **19**, 2911–2923.
Finlay D, Healy V, Furlong F, O'Connell FC, Keon NK and Martin F. (2000). *Cell Death Differ.*, **7**, 303–313.
Gille H, Sharrocks AD and Shaw PE. (1992). *Nature*, **258**, 414–417.
Gioeli D, Mandell JW, Petroni GR, Frierson Jr HF and Weber MJ. (1999). *Cancer Res.*, **59**, 279–284.
Giroux S, Tremblay M, Bernard D, Cardin-Girard JF, Aubry S, Larouche L, Rousseau S, Huot J, Landry J, Jeannotte L and Charon J. (1999). *Curr. Biol.*, **9**, 369–372.
Hagemann C and Rapp UR. (1999). *Exp. Cell Res.*, **253**, 34–46.
Hall-Jackson CA, Eyers PA, Cohen P, Goedert M, Boyle FT, Hewitt NH and Hedge P. (1999). *Chem. Biol.*, **6**, 559–568.
Henry SP, Monteith D, Bennett F and Levin AA. (1997). *Anticancer Drug Des.*, **12**, 409–420.
Her JH, Lakhani S, Zu K, Vila J, Dent P, Sturgill TW and Weber MJ. (1993). *Biochem. J.*, **296**, 25–31.
Herrera R. (1998). *J. Cell Science*, **111**, 1039–1049.
Hill CS and Treisman R. (1995). *Cell*, **80**, 199–211.
Holmstrom TH, Tran SE, Johnson VL, Ahn NG, Chow SC and Eriksson JE. (1999). *Mol. Cell. Biol.*, **19**, 5991–6002.
Hoshino R, Chatani Y, Yamori T, Tsuruo T, Oka H, Yoshida O, Shimada Y, Ari-I S, Wada H, Fujimoto J and Kohno M. (1999). *Oncogene*, **18**, 813–822.
Janknect R, Ernst WH, Pingoud V and Nordheim A. (1993). *EMBO J.*, **12**, 5097–5104.
Kolch W. (2000). *Biochem. J.*, **351**, 289–305.
Kolch W, Heidecker G, Kochs G, Hummel R, Vahidi H, Mischak H, Finkenzeller G, Marme D and Rapp UR. (1993). *Nature*, **364**, 249–252.
Lange CA, Richer JK, Shan T and Horwitz KB. (1996). *J. Biol. Chem.*, **273**, 31308–31316.
Lee SW, Fang L, Igarashi M, Ouchi T, Lu KP and Aaronson SA. (2000). *Proc. Natl. Acad. Sci. USA*, **97**, 8302–8305.
Lenormand P, Brondello JM, Brunet A and Pouyssegur J. (1998). *J. Cell. Biol.*, **142**, 625–633.
Lewis TS, Shapiro PS and Ahn NG. (1998). *Adv. Cancer Res.*, **74**, 49–139.
Li W, Han M and Guan K-L. (2000). *Genes Dev.*, **14**, 895–900.
Lu H-T, Yang DD, Wysk M, Gatti E, Mellman I, Davis RJ and Flavell RA. (1999). *EMBO J.*, **18**, 1845–1857.
Mansour SJ, Matten WT, Hermann AS, Candia JM, Rong S, Fukasawa K, Vande Woude GF and Ahn NG. (1994). *Science*, **265**, 966–970.
Marais R, Wynne J and Treisman R. (1993). *Cell*, **73**, 381–393.
McDonald OB, Chen WJ, Ellis B, Hoffman C, Overton L, Rink M, Smith A, Marshall CJ and Wood ER. (1999). *Anal. Biochem.*, **268**, 318–329.
Milanini J, Vinals F, Pouyssegur J and Pages G. (1998). *J. Biol. Chem.*, **273**, 18165–18172.
Monia BP, Johnston JF, Geiger T, Muller M and Fabbro D. (1996). *Nature Med.*, **2**, 668–675.
O'Dwyer PJ, Stevenson JP, Gallagher M, Cassella A, Vasilevskaya I, Monia BP, Holmlund J, Dorr FA and Yao KS. (1999). *Clin. Cancer Res.*, **5**, 3977–3982.
Pages G, Lenorman D, L'Allemain G, Chambard JC, Meloche S and Pouyssegur J. (1993). *Proc. Natl. Acad. Sci. USA.*, **90**, 8319–8323.
Pages G, Guerin S, Grall D, Bonino F, Smith A, Anjuere F, Auberger P and Pouyssegur J. (1999). *Science*, **286**, 1374–1377.
Pang L, Sawada T, Decker SJ and Saltiel AR. (1995). *J. Biol. Chem.*, **270**, 13585–13588.
Persons DL, Yazlovitskaya EM, Cui W and Pelling JC. (1999). *Clin. Cancer Res.*, **5**, 1007–1014.
Persons DL, Yazlovitskaya EM and Pelling JC. (2000). *J. Biol. Chem.*, in press.
Petit AM, Rak J, Hung MC, Rockwell P, Goldstein N, Fendly B and Kerbel RS. (1997). *Am. J. Pathol.*, **151**, 1523–1530.
Potempa S and Ridley AJ. (1998). *Mol. Biol. Cell.*, **9**, 2185–2200.
Ridley AJ, Comoglio PM and Hall A. (1995). *Mol. Cell Biol.*, **15**, 1110–1122.
Schaeffer HJ, Catling AD, Eblen ST, Collier LS, Krauss A and Weber MJ. (1998). *Science*, **281**, 1668–1671.
Scheid MP, Schubert KM and Duronio V. (1999). *J. Biol. Chem.*, **274**, 31108–31113.
Sebolt-Leopold JS, Dudley DT, Herrera R, Van Becelaere K, Wiland A, Gowan RC, Tecle H, Barrett SD, Bridges A, Przybranowski S, Leopold WR and Saltiel AR. (1999). *Nature Med.*, **5**, 810–816.
Seger R, Ahn NG, Posada J, Munar ES, Jensen AM, Cooper JA, Cobb MH and Krebs EG. (1992). *J. Biol. Chem.*, **267**, 14373–14381.
Stanton Jr VP and Cooper GM. (1987). *Mol. Cell. Biol.*, **7**, 1171–1179.
Stevenson JP, Yao K-S, Gallagher M, Friedland D, Mitchell EP, Cassella A, Monia B, Kwoh TJ, Yu R, Holmlund J, Dorr FA and O'Dwyer PJ. (1999). *J. Clin. Oncol.*, **17**, 2227–2236.
Stewart S, Sundaram M, Zhang Y, Lee J, Han M and Guan K-L. (1999). *Mol. Cell. Biol.*, **19**, 5523–5534.
Stokoe D, Macdonald SG, Cadwallader K, Symons M and Hancock JF. (1994). *Science*, **264**, 1463–1467.
Storm SM and Rapp UR. (1993). *Toxicol. Lett.*, **67**, 201–210.
Tanimura S, Chatani Y, Hoshino R, Sato M, Watanabe S, Kataoka T, Nakamura T and Kohno M. (1998). *Oncogene*, **17**, 57–68.
Wang H-G, Rapp UR and Reed JC. (1996). *Cell*, **87**, 629–638.
Wang X, Martindale JL and Holbrook NJ. (2000). *J. Biol. Chem.*, In press.
Weber CK, Slupsky JR, Herrmann C, Schuler M, Rapp UR and Block C. 2000). *Oncogene*, **19**, 169–176.
Welch DR, Sakamaki T, Pioquinto R, Leonard TO, Goldberg SF, Hon Q, Erikson RL, Rieber M, Rieber MS, Hicks DJ, Bonventre JV and Alessandrini A. (2000). *Cancer Res.*, **60**, 1552–1556.
Yuen AR and Sikic BI. (2000). *Frontiers Biosci.*, D588–593.
Yeung K, Seitz T, Li S, Janosch P, McFerran B, Kaiser C, Fee F, Katsanakis KD, Rose DW, Mischak H, Sedivy JM and Kolch W. (1999). *Nature*, **401**, 173–177.
Yeung K, Janosch P, McFerran B, Rose DW, Mischak H, Sedivy JM and Kolch W. (2000). *Mol. Cell. Biol.*, **20**, 3079–3085.
Yung Y, Yao Z, Hanoch T and Seger R. (2000). *J. Biol. Chem.*, **275**, 15799–15808.
Zecevic M, Catling AD, Eblen ST, Renzi L, Hittle JC, Yen TJ, Gorbsky GJ and Weber MJ. (1998). *J. Cell Biol.*, **142**, 1547–1558.
Zeneca Ltd. (1998). WO9822103.
Zhu X and Assoian RK. (1995). *Mol. Biol. Cell*, **6**, 273–282.

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Cellular Signalling 15 (2003) 455–462
CELLULAR SIGNALLING
www.elsevier.com/locate/cellsig

Review article

Molecular recognitions in the MAP kinase cascades

Takuji Tanoue[1], Eisuke Nishida*

Department of Cell and Developmental Biology, Graduate School of Biostudies, Kyoto University, Sakyo, Kyoto 606-8502, Japan

Received 9 August 2002; accepted 21 September 2002

Abstract

The mitogen-activated protein kinase (MAPK) cascades play a pivotal role in many aspects of cellular functions, and are evolutionarily conserved from yeast to mammals. In mammals, there are four subfamily members in the MAPKs. Each MAPK has its own activators, substrates and inactivators. In order to achieve normal cellular functions, the MAPK cascades should transduce signals with high efficiency and fidelity. However, the molecular basis for the mechanism underlying the specific reactions in the MAPK cascades has not been fully understood. The MAPKs form a globular structure without a distinct domain specific for protein–protein interactions. Recent studies revealed two mechanisms regulating the signalling, the docking interaction and the scaffolding. The docking interaction is achieved through the common docking domain (the CD domain) on MAPKs, and is different from a transient enzyme–substrate interaction through the active centre of the enzymes. Almost all the MAPK-interacting molecules have a conserved motif interacting with the CD domain. The scaffolding usually utilizes a third molecule to tether several components of the MAPK cascades. Both of them are thought to regulate the enzymatic specificity and efficiency.

Keywords: MAP kinase; Phosphorylation; Docking interaction; CD domain; Scaffolding; Specificity

1. Introduction

In a living cell, a large number of molecules should fulfil their specific mission at an appropriate time and place, independently of, or coordinately with, other molecules. To avoid undesirable outcomes, each molecule must react with appropriate partners. Studying the molecular mechanism regulating each specific reaction has only begun in recent years. A specific reaction may be regulated by a specific protein–protein interaction. Many motifs and/or domains for protein–protein interaction have been identified that can help to regulate each specific reaction [1–3].

The MAP kinase (MAPK) cascades convey a signal in the form of phosphorylation events. MAPKs are phosphorylated by MAP kinase kinases (MAPKKs), phosphorylate various targets, such as transcription factors and MAPK-activated protein kinases (MAPKAPKs), and are dephosphorylated and inactivated by several MAPK-phosphatases (MKPs). There are four subgroups in the MAPK family, ERK, p38, JNK/SAPK and ERK5. ERK is activated mainly by mitogenic stimuli, whereas p38 and JNK/SAPK are activated mainly by stress stimuli or inflammatory cytokines [4–13]. ERK5 is activated by EGF, NGF, osmotic stresses and oxidative stresses. Activation of these protein kinases leads to variable responses, such as gene expression, cell proliferation, differentiation, cell cycle arrest, apoptosis and early development, depending on the cell type. Seven members of the MAPKK family, 10 members of the MAPK-activated protein kinase (MAPKAPK) family and 10 members of the dual-specificity MAPK-phosphatase (MKP) family have been reported to date. Each MAPK has several specific MAPKKs, MAPKAPKs and MKPs. Such a variety of molecules must transduce signals with high efficiency and specificity. Then, fine and elegant mechanisms would be required to arrange and maintain such complicated systems. The molecular basis for this accurate signal transduction has been addressed in recent years. There are two main mechanisms regulating the signal transduction in the MAP kinase cascades: the docking interaction and the scaffolding. The docking interaction is achieved through specific conserved regions on MAPKs and MAPK-interacting molecules. Scaffolding generally requires a third molecule to tether enzymes and substrates.

* Corresponding author. Tel.: +81-75-753-4230; fax: +81-75-753-4235.
E-mail address: L50174@sakura.kudpc.kyoto-u.ac.jp (E. Nishida).
[1] Present address: RIKEN Center for Developmental Biology, 2-2-3 Minatojima-Minamimachi, Chuo-ku, Kobe 650-0047, Japan.

0898-6568/02/$ - see front matter © 2002 Elsevier Science Inc. All rights reserved.
doi:10.1016/S0898-6568(02)00112-2

2. Docking sites on MAPK-interacting molecules

MAPKs form a complex with their cognate MAPKKs, substrates and phosphatases [14–47]. The complex formation is distinct from a transient enzyme–substrate interaction through the active centre. For example, a complex formation between MEK1 (a MAPKK specific for ERK) and ERK is achieved through an N-terminal portion of MEK1 outside its catalytic domain [16]. A C-terminal portion outside the catalytic domain of RSK (a MAPKAPK specific for ERK) is required for a complex formation with ERK2 [23,28,38]. The ability to form a complex well correlates with the enzymatic specificity. For example, ERK2 forms a complex with its specific phosphatase, MKP-3 (also known as Pyst1) [17,18], but not with hVH-5, MKP-5 or MKP-7 (MKPs specific for JNK/SAPK and p38) [18,29,40]. ERK forms a complex with RSK but not with MAPKAPK-2 (a MAPKAPK specific for p38) [23,28,38]. Such a complex formation is called docking interaction, which is thought to regulate the enzymatic efficiency and specificity in the MAPK pathways. Recent studies provided cues to understand the molecular nature of the docking interaction. We and others identified a conserved MAPK-docking motif in the primary sequences of MAPK-interacting molecules [23,25,28,33]. The MAPK-docking site is featured by a cluster of positively charged amino acids surrounded by hydrophobic amino acids (Fig. 1). When the positively charged amino acids are replaced by neutral ones, both the ability to bind to the MAPKs and the efficiency of the enzymatic reaction are significantly reduced. Hydrophobic amino acids are also important for the docking interactions [41,42,45]. The N-terminal portion of SEK1/MKK4, a MAPKK for JNK, contains the MAPK-docking site and serves as a binding site for both the upstream activator, MEKK1, and the downstream target JNK [20]. This mechanism may regulate signalling through SEK1/MKK4. The N-terminal portion of MEK1 and the C-terminal portion of RSK2, both of which include the MAPK-docking site, are sufficient for binding to ERK2 [16,23,28,33]. Thus, the docking site appears to be necessary and sufficient for binding to MAPKs. The most important feature of the MAPK-docking site is that it locates outside the catalytic domain of the MAPK-interacting molecules (Fig. 2). Therefore, the docking interactions are different from the transient enzyme–substrate interaction through the active centre during the catalytic reaction.

MAPKK	MEK1	MPKKKPTPIQLNPNP	ERK
	MEK2	MLARRKPVLPALTINP	
	MKK3	KGKSKRKKDLRI	p38
	MKK6	SKGKKRNPGLKIP	
	SEK1/MKK4	QGKRKALKLNF	JNK/SAPK, p38
	MKK7	EARRRIDLNLDISP	JNK/SAPK
	MEK5	LKKSSAELRKIL	ERK5/BMK
MAPKAPK	RSK1	SSILAQRRVRKLPSTTL	ERK
	RSK2	RSTLAQRRGIKKITSTAL	
	RSK3	SSNLAQRRGMKRLTSTRL	
	MNK2	QSKLAQRRQRASLSATPV	
	MNK1	KSRLARRRALAQAGRSRD	ERK, p38
	MSK1	KAPLAKRRKMKKTSTSTE	
	MAPKAPK2	NPLLLKRRKKARALEAAA	p38
	MAPKAPK3	NRLLNKRRKKQAGSSSAS	
	PRAK	NNPILRKRKLLGTKPKDS	
	RSKB	NAPLAKRRKQKLRSATAS	
PTP	EC-PTP	GLQERRGSNVSLTLDM	ERK
	HePTP	RLQERRGSNVALMLDV	
	STEP	GLQERRGSNVSLTLDM	
MKP	CL100/MKP-1	RFSTIVRRRAKGAKGAG	(ERK), p38, JNK/SAPK
	MKP-2	RCNTIVRRRAKGSVSLE	
	PAC1	PWNALLRRRARARGPP	
	MKP-3	PGIMLRRLQKGNLPVR	ERK
	Pyst2	PGLMLRRLRKGNLPIR	
	MKP-4	LPALLLRRLRRGSLSVR	
	B23	LNSVVLRRARGGAVSA	
	hVH5	SKLVKRRLQQGKVTI	p38, JNK/SAPK
	MKP-5	DKISRRRLQQGKITV	
	MKP-7	SKLMKRRLQQDKVLI	
transcription factors	MEF2A	NSRKPDLRVVIPPSSK	p38
	SAP-1	RSKKPKGLGLAPTLVIT	ERK,p38
	Elk-1	KGRKPRDLELPLSPSLL	

Fig. 1. The MAPK-docking site is conserved among the MAPK-interacting molecules. Note that a cluster of positively charged amino acid residues is surrounded by hydrophobic amino acids. The MAPK-docking site in transcription factors is often called the D domain.

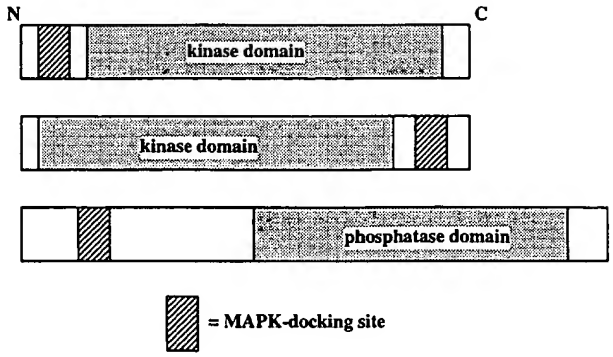

Fig. 2. The MAPK-docking site locates outside the catalytic domain of MAPKKs (upper), MAPKAPKs (middle) and MKPs (lower). The MAPK-docking site of PTP also locates outside the catalytic domain (not shown).

3. Docking sites in MAPK substrates other than MAPKAPKs

Transcription factors regulated by MAPKs, such as Elk-1, Sap1 and MEF2, also have a region resembling the MAPK-docking site identified in MAPKKs, MKPs and MAPKAPKs [21,22,30,36,43] (Fig. 1). The region is often called the D domain. The D domain is required for efficient phosphor-

14217030 PMID: 12639709

Mechanisms of regulating the Raf **kinase family.**

Chong Huira; Vikis Haris G; Guan Kun-Liang

Department of Biological Chemistry, University of Michigan, Ann Arbor, MI 48109, USA.

Cellular signalling (England) May 2003, 15 (5) p463-9, ISSN 0898-6568--Print Journal Code: 8904683

Contract/Grant No.: 5T32 GM 07544; GM; NIGMS

Publishing Model Print

Document type: Journal Article; Review

Languages: ENGLISH

Main Citation Owner: NLM

Record type: MEDLINE; Completed

Subfile: INDEX MEDICUS

The **MAP Kinase pathway** is a key signalling mechanism that regulates many cellular functions such as cell growth, transformation and apoptosis. One of the essential components of this pathway is the serine/threonine kinase, **Raf** . **Raf** (MAPKK kinase, MAPKKK) relays the extracellular signal from the receptor/ **Ras** complex to a cascade of cytosolic kinases by phosphorylating and activating MAPK/ERK kinase (MEK; MAPK kinase, MAPKK) that phosphorylates and activates extracellular signal regulated kinase (ERK; mitogen-activated protein kinase, MAPK), which phosphorylates various cytoplasmic and nuclear proteins. Regulation of both **Ras** and **Raf** is crucial in the proper maintenance of cell growth as oncogenic mutations in these genes lead to high transforming activity. **Ras** is mutated in 30% of all human cancers and B- **Raf** is mutated in 60% of malignant melanomas. The mechanisms that regulate the small GTPase **Ras** as well as the downstream kinases MEK and extracellular signal regulated kinase (ERK) are well understood. However, the regulation of **Raf** is complex and involves the integration of other signalling pathways as well as intramolecular interactions, phosphorylation, dephosphorylation and protein-protein interactions. From studies using mammalian isoforms of **Raf** , as well as C. elegans lin45- **Raf** , common patterns and unique differences of regulation have emerged. This **review** will summarize recent findings on the regulation of **Raf** kinase. (88 Refs.)

Descriptors: *Proto-Oncogene Proteins c- **raf** --metabolism--ME; Animals; Macromolecular Substances; Monomeric GTP-Binding Proteins--metabolism--ME; Phosphorylation; Protein Structure, Tertiary; Proto-Oncogene Proteins A- **raf** ; Proto-Oncogene Proteins B- **raf** ; Proto-Oncogene Proteins c- **raf**0 --chemistry--CH; Research Support, Non-U.S. Gov't; Research Support, U.S. Gov't, P.H.S.

CAS Registry No.: 0 (Macromolecular Substances)

Enzyme No.: EC 2.7.1.37 (Proto-Oncogene Proteins A- **raf**); EC 2.7.1.37 (Proto-Oncogene Proteins B- **raf**); EC 2.7.1.37 (Proto-Oncogene Proteins c- **raf**); EC 3.6.5.2 (Monomeric GTP-Binding Proteins)

Record Date Created: 20030317

Record Date Completed: 20031014

9/9/8

DIALOG(R)File 155:MEDLINE(R)

ylation by MAPKs. PDE also has a MAPK-docking site and is regulated by MAPKs. We have recently identified Tob as a novel substrate of MAPKs [48]. Tob is a member of the Tob and BTG anti-proliferative protein family. Tob has a MAPK-docking site in its N-terminal portion and an FXFP motif (see below) in its C-terminal portion. Although it is not known whether all the MAPK-interacting molecules, identified or unidentified, have a MAPK-docking site or the D domain, existence of such a region might serve as one of the criteria for being a MAPK-interacting molecule. The D domain was found to form a modular structure (see Fig. 4B). A basic region is followed by an LXL motif and a hydrophobic region. While ERK phosphorylation requires all of the three modules, p38 can phosphorylate a substrate without the second module, the LXL motif [43].

4. Specificity determinants in the MAPK-docking site

The docking sites for p38 have more consecutive positively charged amino acids than those for ERK (see Fig. 1). However, adding or deleting positively charged amino acids in the docking sites does not result in conversion of the docking specificity of the molecules. An exchange of the docking site between ERK-interacting molecules and p38-interacting molecules usually failed to convert the specificity of the molecules, although a successful example was reported, where the conversion of the C-terminal portion, the MAPK-docking site, of RSK2 to that of MAPKAPK2 results in the conversion of the specificity [38]. RSK2 is an ERK-specific MAPKAPK, and MAPKAPK2 is a p38-specific MAPKAPK. A chimeric protein of RSK2 containing the MAPK-docking site of MAPKAPK2 can be activated by p38, but not by ERK. For most proteins, it is difficult to change their binding specificity. It was often observed that mutations introduced in the binding surface result in either neutral phenotypes or a general loss in the binding. This may be interpreted as follows. The high-affinity binding may generally require efficient exploitation of the multiple potential interactions available on the binding partner, in which each amino acid involved in each interaction is appropriately enmeshed in the docking surface of the binding partners. Then, mutations designed to introduce new interactions may often result in disruption of the binding because they may induce distortion in the appropriate configuration of each amino acids involved. It was reported that multiple regions of ERK2 and p38 are important for the determination of enzymatic specificity of upstream kinases (MAPKKs) towards MAPKs [46]. Recently, it has been shown that the substitution of amino acid residues within the T-loop of p38β2 greatly affects the activatability by MKK3 [47], confirming the importance of the transient enzyme–substrate interaction in determining the substrate specificity. Collectively, both the docking interaction and the transient enzyme–substrate interaction cooperatively regulate the enzymatic specificity in the MAP kinase cascade. Detailed mechanisms should be elucidated in the future studies.

5. The CD domain and the docking groove on the MAP kinases

We have identified a docking site on MAPKs that is featured by a cluster of negatively charged amino acids in the steric structure on the opposite side from the active centre of the molecules and is located in the C-terminal portion of MAPKs in the primary sequence [33] (Fig. 3). When the negatively charged amino acids in this site are replaced by neutral ones, the docking interactions are

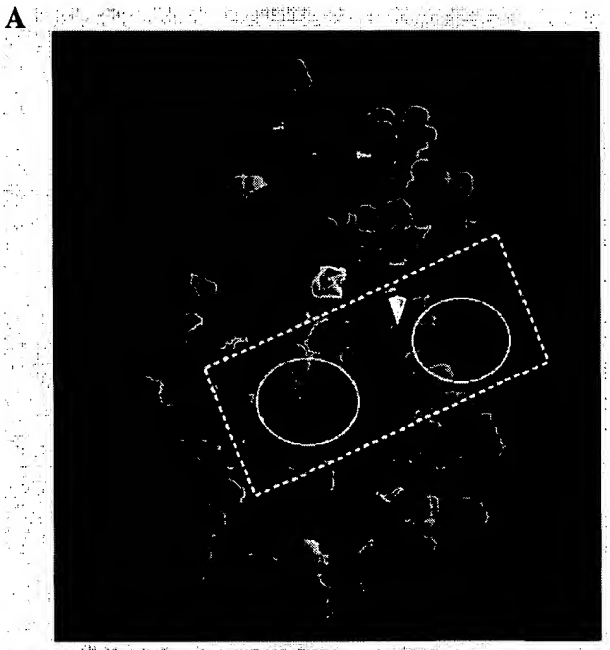

Fig. 3. The docking site on MAPK. (A) The docking groove of ERK2. A dotted box indicates the docking groove. A yellow circle indicates the CD domain. A white circle indicates the ED site. Note that the docking groove locates opposite the active centre. The arrow indicates Asp 160 of ERK2. This diagram was made using RasMol, based on the crystallographic data. (B) The CD domain is conserved in the MAPK family. Amino acid residues indicated by red boxes are negatively charged amino acids, which are expected to be exposed to the surface of the molecules.

disrupted. As this site is commonly used in the docking interactions with activators, substrates and inactivators, we named it the common docking (CD) domain. As the docking interactions of MAPKs with MAPKKs, phosphatases and substrates are mutually exclusive, these docking interactions through the CD domain might regulate the serial signal transduction of the MAPK cascade reactions. Because the MAPK-docking sites of the MAPK-interacting molecules are featured by the positively charged amino acids and the CD domain is composed of negatively charged amino acids, the electrostatic interaction may be important in the docking interactions. The CD domain solely, however, does not determine the docking specificity [33]. In search of another site on MAPKs that might regulate the docking specificity, we identified then a site near the CD domain in the steric structure on MAPKs, and called it the ED site [39] (Fig. 3A). When both the CD domain and the ED site of ERK2 are engineered to mimic those of p38, the docking specificity is converted to the p38-type in the case of docking to several MAPKAPKs and MKPs. We proposed thus a concept of a docking groove, which is composed of the CD domain, the ED site and the surrounding amino acids. While the CD domain is commonly important for every docking interaction, the ED site is differently utilized [39] (Fig. 3A). The molecules specific for p38 MAPK have more consecutive positively charged amino acids in their MAPK-docking site than those specific for ERK. Correspondingly, p38 MAPK has more consecutive negatively charged amino acids in the docking groove than ERK. These are consistent with our docking groove model.

A recent paper reported crystallographic data of a complex between p38 MAPK and each peptide of the MAPK-docking site of MKK3b, a MAPKK specific for p38 MAPK, and that of MEF2A, a transcription factor regulated by p38 MAPK [51]. As discussed above, the MAPK-docking sites consist of charged residues and hydrophobic residues. The data showed that hydrophobic residues of these MAPK-docking sites interact with a hydrophobic groove of p38 MAPK. The hydrophobic groove locates near the ED site. In their crystallographic data, positively charged residues, which are presumed to interact with the CD domain, were not visible. As the electrostatic interaction with the CD domain occurs in the context of the full-length proteins, it is possible that the disorder of the positively charged residues of the MAPK-docking peptides might be caused by the existence of multiple negatively charged residues in the docking groove of p38, such as Glu81 and Asp125, in addition to Asp313, Asp315 and Asp316 in the CD domain, available for electrostatic interactions with the positively charged residues of the MAPK-docking peptides.

6. The sevenmaker mutant in *Drosophila*

Asp 319 (in rat ERK2) in the CD domain is mutated to Asn in the sevenmaker mutant of *Drosophila* ERK/Rolled, which showed a gain-of-function phenotype [49,50]. The phenotype might depend on a balance in the intracellular amounts of activators, inactivators and substrates of ERK and on the properties of their enzymatic reactions. It has recently been reported that a mutation of Asp 160 (in rat ERK2) to Asn leads to a phenotype similar to that of the sevenmaker mutant [52], suggesting involvement of Asp 160 in the docking interaction. Asp 160 is located adjacent to Thr 158 of the ED (TT) site in the steric structure of ERK2 (Fig. 3A). The corresponding amino acid in p38 is Glu 163. It is clear that both Asp 160 of ERK2 and Glu 163 of p38 are located within the docking groove and expected to be exposed to the surface of the molecules [53–57]. Therefore, this recent report is consistent with our idea of the docking groove. The CD domain is conserved from yeast to humans. In yeast, a Hog1 mutant, in which a residue corresponding to the sevenmaker mutation of *Drosophila* was mutated, showed a reduced ability to be inactivated by PTP2, a member of the MKP family in yeast [31].

7. The docking surface on MKPs

We have identified a docking surface on MKPs corresponding to the docking groove of MAPKs [45]. The docking surface of MKPs, which includes the previously identified docking site, helps to regulate the docking specificity towards MAPKs. A recent paper reported the solution structure of the N-terminal domain (rhodanese-fold) of MKP-3, in which the docking surface exists [58]. Their results with 2D ^{13}N-edited transverse relaxation-optimized spectroscopy (TROSY) showed that the docking surface region of MKP-3 constitutes a direct interaction surface for binding to ERK2. The docking surface consists of three modules, and each of them regulates the docking specificity towards the members of the MAPK [45]. The region on other MAPK-interacting molecules corresponding to the docking groove should be elucidated in future studies.

8. Docking interactions regulate subcellular localization of the molecules in the MAPK cascades

The docking interactions may also function to regulate subcellular localization of MAPKs and MAPK-interacting molecules. For example, the N-terminal portion of MEK1 is the ERK-docking domain, and MEK1 can function as a cytoplasmic anchor for ERK2 [16]. PTP-SL has also been shown to be a cytoplasmic anchor for ERK [27]. Furthermore, a form of ERK1, in which the region near the CD domain is deleted or spliced out, was found to show different subcellular localization from wild-type ERK, which might be caused by the loss of associations with the interacting molecules, including MEK1 and PTPs [59,60].

A

	substrates	FXFP motif
Ets transcription factors	lin-1 (C. elegans)	QVFQFPPV
	Elk-1 (H. sapiens)	LSFQFPSS
	SAP-1 (H. sapiens)	TLFQFPSV
	Yan (D. melanogaster)	DKFQFHPL
Ksr	ksr-1 (C. elegans)	PAFQFPDT
	ksr-1 (Mus musculus)	KKFLFPDT
	Ksr (D. melanogaster)	SRFSFPDI
		SQFNFPNV
PDE	PDE4D (H. sapiens)	EKFQFELT

B

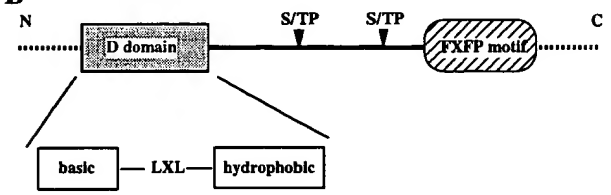

Fig. 4. The FXFP motif. (A) The FXFP motif is identified in the Ets transcription factors, KSR, and PDE. The FXFP motif is conserved across the species. (B) The schematic representative of the Ets transcription factors is shown. S/TP indicates the MAPK-phosphorylation site. The S/TP site is sandwiched by the D domain and the FXFP motif. The D domain forms a modular structure.

In addition, it was shown that the docking interaction with p38 regulates the subcellular localization of MAPKAPK-3, a member of MAPKAPKs [39]. The p38-docking site of MAPKAPK-3 overlaps the nuclear localization signal. Thus, the docking of p38 to MAPKAPK-3 inhibits the NLS function. The p38-docking site of MSK2 was also shown to function as NLS [61].

9. The FXFP motif on transcription factors

There exists another conserved motif (the FXFP motif) on the substrates of MAPKs, mainly on the transcription factors, such as Elk-1 and SAP1, that is required for efficient phosphorylation by MAPKs [25,35]. The motif is featured by two phenylalanines separated by one amino acid, and followed by proline (Fig. 4A). The site exists in the portion C-terminal to a MAPK-phosphorylation site (Fig. 4B). When either one or both of the phenylalanines were replaced by alanines, the efficiency of the phosphorylation was significantly reduced. Importantly, in *C. elegans*, gain-of-function mutants of lin-1, a member of Ets family transcription factors negatively regulated by MAPKs, were identified, in which the FXFP site was mutated. This suggests that the FXFP motif is physiologically important [25]. Although the FXFP motif was originally identified as an ERK-specific recognition motif, a recent report shows that the motif on SAP1 is required for efficient phosphorylation by both ERK and p38 [36]. The FXFP motif is also identified on KSR and PDE, both of which are known to be substrates of MAPKs [35,62].

10. Prediction of a site on MAPKs corresponding to the FXFP motif

The FXFP site might protrude from the neighbouring amino acids, and a site on MAPKs corresponding to the FXFP site might form a hydrophobic pocket. The FXFP motif is effective even when a MAPK-phosphorylation site locates only five amino acids upstream to the motif [25,35,36]. This means that the site on MAPKs corresponding to the FXFP site locates near the active centre of MAPKs. In addition, the relative direction and configuration of the amino acid residues of substrates around a phosphorylated serine/threonine to the active centre of MAPKs are predicted [53–57]. Based on these assumptions, we predict sites on MAPKs for the FXFP motif (Fig. 5). There are three candidates. The identification of the site on MAPKs corresponding to the FXFP motif may help our understanding of the molecular mechanism regulating the specific recognition of substrates by MAPKs.

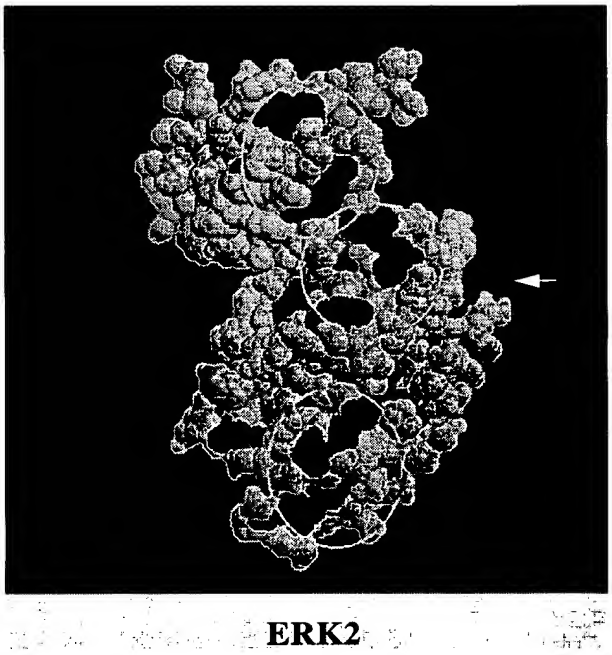

Fig. 5. Prediction of a site on ERK2 corresponding to the FXFP motif. Hydrophobic amino acid residues are coloured by green, dark green or grey. A red asterisk indicates the active centre of ERK2. There are three candidates for a region corresponding to the FXFP motif (yellow circles). The white arrow indicates the CD domain. This diagram was made using RasMol, based on the crystallographic data [55].

9/9/3
DIALOG(R)File 155:MEDLINE(R)

15607082 PMID: 16170185

Raf : **a strategic target for therapeutic development against cancer.**

Beeram Muralidhar; Patnaik Amita; Rowinsky Eric K
University of Texas Health Science Center at San Antonio, San Antonio, TX, USA.

Journal of clinical oncology - official journal of the American Society of Clinical Oncology (United States) Sep 20 2005, 23 (27) p6771-90, ISSN 0732-183X--Print Journal Code: 8309333
Publishing Model Print
Document type: Journal Article; Review
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
Subfile: INDEX MEDICUS

The mitogen-activated protein **kinase** (**MAPK**) signaling **pathway** plays a critical role in transmitting proliferative signals generated by cell surface receptors and cytoplasmic signaling elements to the nucleus. Several important signaling elements of the MAPK pathway, particularly **Ras** and **Raf** , are encoded by oncogenes, and as such, their structures and functions can be modified, rendering them constitutively active. Because the MAPK pathway is dysregulated in a notable proportion of human malignancies, many of its aberrant and critical components represent strategic targets for therapeutic development against cancer. **Raf** , which is an essential serine/threonine **kinase** constituent of the **MAPK** **pathway** and a downstream effector of the central signal transduction mediator **Ras** , is activated in a wide range of human malignancies by aberrant signaling upstream of the protein (eg, growth factor receptors and mutant **Ras**) and activating mutations of the protein itself, both of which confer a proliferative advantage. Three isoforms of **Raf** have been identified, and therapeutics targeting **Raf** , including small-molecule inhibitors and antisense oligodeoxyribonucleotides (ASON), are undergoing clinical evaluation. The outcomes of these investigations may have far-reaching implications in the management of many types of human cancer. This **review** outlines the structure and diverse functions of **Raf** , the rationale for targeting **Raf** as a therapeutic strategy against cancer, and the present status of various therapeutic approaches including ASONs and small molecules, particularly sorafenib (BAY 43-9006). (236 Refs.)

Descriptors: *Antineoplastic Agents--administration and dosage--AD; *Drug Delivery Systems; *MAP Kinase Signaling System--physiology--PH; *Neoplasms --drug therapy--DT; *Neoplasms--genetics--GE; * **raf** Kinases--metabolism --ME; Animals; Benzenesulfonates--administration and dosage--AD; Comparative Study; Down-Regulation; Humans; MAP Kinase Kinase 1--genetics --GE; MAP Kinase Kinase 1--metabolism--ME; Molecular Biology; Mutation; Neoplasms--physiopathology--PP; Proto-Oncogene Proteins--genetics--GE; Proto-Oncogene Proteins--metabolism--ME; Proto-Oncogene Proteins A- **raf** --genetics--GE; Proto-Oncogene Proteins A- **raf** --metabolism--ME; Proto-Oncogene Proteins B- **raf** --genetics--GE; Proto-Oncogene Proteins B- **raf** --metabolism--ME; Proto-Oncogene Proteins c- **raf** --genetics--GE; Proto-Oncogene Proteins c- **raf** --metabolism--ME; Pyridines--administration and dosage--AD; Research Support, Non-U.S. Gov't; Sensitivity and Specificity; Signal Transduction; **raf** Kinases--genetics--GE

CAS Registry No.: 0 (4-(4-(3-(4-chloro-3-trifluoromethylphenyl)ureido)phenoxy)pyridine-2-carboxyllic acid methyamide-4-methylbenzenesulfonate); 0 (Antineoplastic Agents); 0 (Benzenesulfonates); 0 (MAP Kinase Signaling System); 0 (Proto-Oncogene Proteins); 0 (Pyridines)

Enzyme No.: EC 2.7.1.- (MAP Kinase Kinase 1); EC 2.7.1.37

11. Difference between the interaction achieved through the CD domain and that through the FXFP motif

The interaction achieved through the FXFP motif is also called docking interaction. However, it is clear that the FXFP motif is different from the MAPK-docking site corresponding to the docking groove. The former exists always downstream to the phosphorylation site in the primary sequence, but the latter exists on either side. While the docking site exists in MAPKKs, MKPs, MAPKAPKs and several other substrates, the FXFP motif is reported only for substrates other than MAPKAPKs, such as Elk-1 and Sap-1. Elk-1, Sap-1 and MEF2 transcription factors have another site (the D domain) (see Fig. 4) that resembles the docking site corresponding to the CD domain [21,22,30,36,43]. The D domain is also needed for efficient phosphorylation by MAPKs. Whether the D domain also interacts with the CD domain should be determined.

12. The LXLXXXF motif

A recent study reported that another hydrophobic motif (LXLXXXF) serves as a MAPK-docking site [44]. The Ets family has 25 members in human, and commonly shares a DNA-binding ETS domain [63]. A subset of the family has the pointed (PNT) domain, which serves as a repressor or an enhancer of transcription. The PNT domain shows a similar fold, but divergent surface chemistry. The LXLXXF motif resides in a subset of the PNT domain, which serves as a MAPK-docking site, and explains the diversity of the functions of the domain. The LXLXXXF motif of PNT domain of Ets-1 and Ets-2 forms a hydrophobic patch on the molecules. A site on ERK corresponding to the LXLXXXF motif is also predicted to be a hydrophobic patch [44].

13. Scaffold proteins

In budding yeast, Ste5p is known to function as a scaffold protein, which regulates the specificity of two different signalling pathways; the mating pathway and the invasive growth pathway [64,65]. Ste5p is required for the former. Mating is mediated by a G-protein-coupled receptor. The MAPK cascade for mating consists of Ste20p (MAPKKKK), Ste11p (MAPKKK), Ste7p (MAPKK) and Fus3p (MAPK). Except for Fus3p MAPK, the components are also utilized in the invasive growth pathway, which activates Kss1p MAPK. Ste5p associates with all the components of the mating MAPK cascade including G protein to prevent inappropriate activation of Kss1p MAPK under stress stimuli. To date, no mammalian protein homologous to Ste5p was identified.

In mammals, the JNK-interacting protein (JIP) family is a well-known scaffold protein [12,66–71]. The JIP family consists of three members, JIP1, 2 and 3. JIP1 and JIP2 are ~ 50% identical, have a similar domain structure and form homodimeric and heterodimeric complexes. JIP3 has no homology with JIP1 or 2. The JIP proteins bind to JNK, MKK7 and members of the mixed-lineage protein kinase (MLK) group (Fig. 6B). In biochemical assays and co-transfection assays, the JIP proteins have been suggested to be scaffold proteins [12,66–71]. Recently, Whitmarsh et al. [71] created mice carrying a targeted deletion of the JIP1 gene, and showed that JIP1 is required for stress-induced activation of JNK in hippocampal neurons. Sunday Driver (Syd), a drosophila homologue of JIP3 protein, was recently identified [72]. Syd associates with kinesin-1, *Drosophila* kinesin light chain, and mutations in Syd and kinesin-1 cause similar phenotypes such as aberrant accumulation of axonal cargoes. In *C. elegans*, UNC-16 encodes a homologue of JIP3 and Syd [73]. Partial loss-of-function mutations in UNC-16 result in mislocalization of synaptic vesicle and glutamate receptor. UNC-16 binds to JNK 1, JKK-1 and SEK1. Mutations in JNK-1 or JKK1 also result in synaptic vesicle mislocalization. Mammalian JIP1, 2 and 3 were also found to interact with the kinesin light chain [74]. These reports raise the possibility that JIP is a substrate of JNK. How the JNK pathway participates in synaptic vesicle trafficking is currently unknown. Additionally, JIP1 and 2 have been reported to associate with RhoGEF and ApoER2, a receptor for Reelin, although the physiological meaning of these interactions has not been determined [75,76].

β-arrestin has recently been shown to be a scaffold for the JNK pathway [77]. When G-protein-coupled receptors

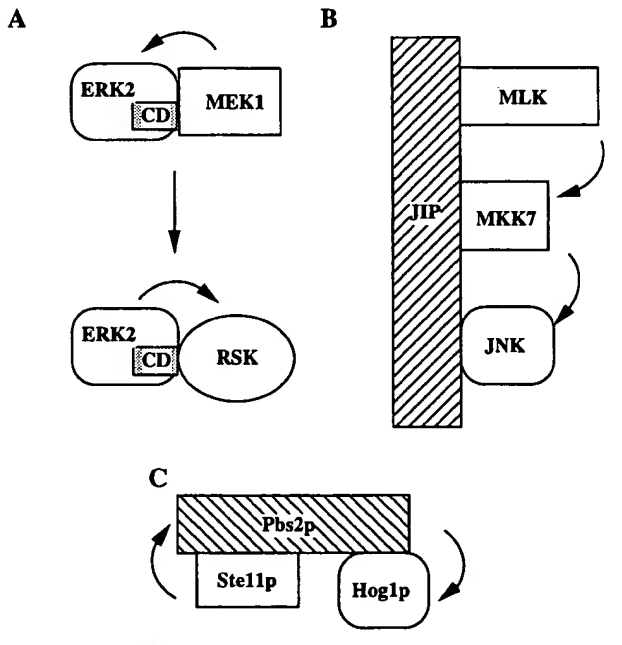

Fig. 6. In the MAPK cascades, two kinds of mechanisms for the recognition of the molecule are used. The docking interaction is achieved through the docking groove including the CD domain. There are two kinds of scaffolding. In B, a third molecule is required. In C, an enzyme serves as a scaffold for assembling the other enzymes.

(GPCRs) bind to ligand, they are phosphorylated by GPCR kinases. The phosphorylated GPCRs recruit β-arrestin2, which serves as a scaffold to assemble the components of the JNK pathway [77]. β-arrestin2, not β-arrestin1, binds to JNK3 and ASK1, a member of MAPKKK, and indirectly binds to SEK1/MKK4. Importantly, β-arrestin2 enhances the activation of JNK3 by ASK1 in a co-expression assay. Thus, β-arrestin serves as a scaffold protein for the JNK pathway regulated by seven transmembrane-spanning receptors. β-arrestins were first discovered as molecules for desensitization of GPCRs, and were also known to act as adaptors to facilitate clathrin-mediated endocytosis of certain members of the GPCR family [78]. Thus, like the JIP family, β-arrestin2 might also have multiple functions.

KSR is also known to associate with several components of the MAPK cascade, including Raf-1, MEK1/2 and ERK1/2 [79]. KSR was originally identified as a gene responsible for suppressing the phenotype of the gain-of-function mutant of Ras [80]. As KSR has a kinase-like domain (the C5 domain), which is very similar to the kinase domain of Raf-1 MAPKKK, it was originally thought of as a member of the MAPKKK family. However, the mammalian homologues have an arginine residue instead of a conserved lysine residue in kinase subdomain II that is thought to be critical for phosphotransferring reaction. Recent studies indicate that it functions as a scaffold protein for the Ras pathway rather than a kinase [79,81,82]. A dsRNA interference technique showed that KSR was necessary for the Ras-dependent ERK activation in cells [82]. The precise mechanism for functioning as a scaffold protein should be elucidated.

MP-1 was identified in a yeast two-hybrid screen with MEK1 [83]. MP-1 was reported to associate with both MEK1 and ERK1, and was suggested to be a scaffold protein. It was found to bind selectively to ERK1, not to ERK2.

In mammals, the specificity of the enzymatic reaction between MAPKKs and MAPKs is stringently determined even in vitro, except for SEK1/MKK4. For example, MKK6, a p38-specific MAPKK, is unable to activate ERK or JNK in vitro, even when excess amounts of proteins are used. The efficient kinase reaction in vitro or in a co-expression system does not necessarily require a third molecule. Thus, scaffold proteins are not always required for the reactions in the MAPK cascades. Scaffold proteins are necessary for several specific cellular functions, as is the case with β-arrestin2.

14. Scaffolding without a third molecule

The strategy utilized by the osmotic stress pathway in yeast does not require a third molecule as a scaffold protein. In yeast, the mating and osmotic stress pathways share a common MAPKKK, Ste11p [64]. Ste11p can activate both Ste7p and Pbs2p, members of MAPKK. In the osmotic stress pathway, Pbs2p activates Hog1p MAPK, a relative of mammalian p38. Sho1p, a transmembrane osmotic sensor, activates Ste11p. Pbs2p has a large N-terminal stretch outside its kinase domain, through which components of the osmotic stress pathway, Sho1p, Ste11p and Hog1p, associate with Pbs2p (see Fig. 6C). Thus, Pbs2p prevents Ste11p from activating Ste7p when osmotic stress is sensed by Sho1p [64]. In mammals, MEKK1, a member of MAPKKK, binds to JNK and ERK2 through its N-terminal portion [84,85]. SEK1/MKK4, a member of MAPKK specific for JNK/SAPK, binds to MEKK1 through the kinase domain of MEKK1. MEK1, an ERK-specific MAPKK, also binds to the N-terminal portion of MEKK1. Physiological roles of these interactions should be elucidated in future studies.

15. Concluding remarks

The MAPK cascade has been intensively studied by a number of investigators over a decade, and these studies provided us with several models that help us to understand other signal transduction systems. However, mechanisms regulating the efficiency and fidelity of the MAPK signalling cascades have not been fully understood. A recent discovery of two important concepts, the docking interaction and the scaffolding, provided cues to understanding the mechanisms (see Fig. 6). In both systems, many questions are yet to be solved.

Acknowledgements

We are especially grateful to Dr. M. Adachi for his valuable suggestions and experiments. We also thank M. Maekawa and T. Yamamoto for their help in preparation of the figures.

References

[1] Pawson T. Nature 1995;373:573–80.
[2] Hunter T. Cell 2000;100:113–27.
[3] Pawson T, Nash P. Genes Dev 2000;14:1027–47.
[4] Sturgill TW, Wu J. Biochim Biophys Acta 1991;1092:350–7.
[5] Ahn NG, Seger R, Krebs EG. Curr Opin Cell Biol 1992;4:992–9.
[6] Nishida E, Gotoh Y. Trends Biochem Sci 1993;18:128–31.
[7] Marshall CJ. Cell 1995;80:179–85.
[8] Kyriakis JM, Avruch J. Physiol Rev 2001;81:807–69.
[9] Treisman R. Curr Opin Cell Biol 1996;8:205–15.
[10] Robinson MJ, Cobb MH. Curr Opin Cell Biol 1997;9:180–6.
[11] Schaeffer HJ, Weber MJ. Mol Cell Biol 1999;19:2435–44.
[12] Davis RJ. Cell 2000;103:239–52.
[13] Chang L, Karin M. Nature 2001;410:37–40.
[14] Bardwell L, Thorner J. Trends Biochem Sci 1996;21:373–4.
[15] Kallunki T, Deng T, Hibi M, Karin M. Cell 1996;87:929–39.
[16] Fukuda M, Gotoh Y, Nishida E. EMBO J 1997;16:1901–8.
[17] Camps M, Nichols A, Gillieron G, Antonsson B, Muda M, Chabert C, et al. Science 1998;280:1262–5.

[18] Muda M, Theodosiou A, Gillieron C, Smith A, Chabert C, Camps M, et al. J Biol Chem 1998;273:9323–9.
[19] Pulido R, Zuniga A, Ullrich A. EMBO J 1998;17:7337–50.
[20] Xia Y, Karin M. Genes Dev 1998;12:3369–81.
[21] Yang SH, Yates PR, Whitmarsh AJ, Davis RJ, Sharrocks AD. Mol Cel Biol 1998;18:710–20.
[22] Yang SH, Whitmarsh AJ, Davis RJ, Sharrocks AD. EMBO J 1998; 17:1740–9.
[23] Gavin AC, Nebreda AR. Curr Biol 1999;9:281–4.
[24] Holland PM, Cooper JA. Curr Biol 1999;9:329–31.
[25] Jacobs D, Glossip D, Xing H, Muslin AJ, Kornfeld K. Genes Dev 1999;13:163–75.
[26] Karim FD, Rubin GM. Mol Cell 1999;3:741–50.
[27] Blanco-Aparicio C, Torres J, Pulido R, J Cell Biol 1999;147:1129–36.
[28] Smith JA, Poteet-Smith CE, Malarkey K, Sturgill TW. J Biol Chem 1999;27:2893–8.
[29] Tanoue T, Moriguchi T, Nishida E. J Biol Chem 1999;274:19949–56.
[30] Yang SH, Galanis A, Sharrocks AD. Mol Cell Biol 1999;19:4028–38.
[31] Zhan XL, Guan KL. Genes Dev 1999;13:2811–27.
[32] Zuniga A, Torres J, Ubeda J, Pulido R. J Biol Chem 1999;274: 21900–7.
[33] Tanoue T, Adachi M, Moriguchi T, Nishida E. Nat Cell Biol 2000; 2:110–6.
[34] Chen P, Hutter D, Yang X, Gorospe M, Davis RJ, Liu YJ. J Biol Chem 2001;276:29440–9.
[35] Fantz DA, Jacobs D, Glossip D, Kornfeld K. J Biol Chem 2001; 276:27256–65.
[36] Galanis A, Yang SH, Sharrocks AD. J Biol Chem 2001;276:965–73.
[37] Slack DN, Seternes OM, Gabrielsen M, Keyse SM. J Biol Chem 2001;276:16491–500.
[38] Smith JA, Poteet-Smith CE, Lannigan DA, Freed TA, Zoltoski AJ, Sturgill TW. J Biol Chem 2001;275:31588–93.
[39] Tanoue T, Maeda R, Adachi M, Nishida E. EMBO J 2001;20:466–79.
[40] Tanoue T, Yamamoto T, Maeda R, Nishida E. J Biol Chem 2001; 276:26629–39.
[41] Xu B, Stippec S, Robinson FL, Cobb MH. J Biol Chem 2001;276: 26509–15.
[42] Zhou B, Wu L, Shen K, Zhang J, Lawrence DS, Zhang Z. J Biol Chem 2001;276:6506–15.
[43] Barsyte-Lovejoy D, Galanis A, Sharrocks AD. J Biol Chem 2002; 277:9896–903.
[44] Seidel JJ, Graves BJ. Genes Dev 2002;16:127–37.
[45] Tanoue T, Yamamoto T, Nishida E. J Biol Chem 2002;277:22942–9.
[46] Brunet A, Pouyssegur J. Science 1996;272:1652–5.
[47] Enslen H, Brancho DM, Davis RJ. EMBO J 2000;19:1301–11.
[48] Maekawa M, Nishida E, Tanoue T. J Biol Chem 2002;277:37783–7.
[49] Brunner D, Oellers N, Szabad J, Biggs WH, Zipursky SL, Hafen E. Cell 1994;76:875–88.
[50] Bott CM, Thorneycroft SG, Marshall CJ. FEBS Lett 1994;352:201–5.
[51] Chang C-I, Xu B, Akella R, Cobb MH, Goldsmith EJ. Mol Cell 2002;9:1241–9.
[52] Lim YM, Nishizawa K, Nishi Y, Tsuda L, Inoue YH, Nishida Y. Genetics 1999;153:763–71.
[53] Zhang F, Strand A, Robbins D, Cobb MH, Goldsmith EJ. Nature 1994;367:704–11.
[54] Wilson KP, Fitzgibbon MJ, Caron PR, Griffith JP, Chen W, McCaffrey PG, et al. J Biol Chem 1996;271:27696–700.
[55] Canagarajah BJ, Khokhlatchev A, Cobb MH, Goldsmith E. Cell 1997;90:859–69.
[56] Wang Z, Canagarajah BJ, Boehm JC, Kassisa S, Cobb MH, Young PR, et al. Proc Natl Acad Sci U S A 1997;94:2327–32.
[57] Xie X, Gu Y, Fox T, Coll JT, Fleming MS, Markland W, et al. Structure 1998;6:983–91.
[58] Farooq A, Chaturvedi G, Mujtaba S, Plotnikova O, Zeng L, Dhalluin C, et al. Mol Cell 2001;7:387–99.
[59] Rubenfeld H, Hanoch T, Seger R. J Biol Chem 1999;274:30349–52.
[60] Yung Y, Yao Z, Hanoch T, Seger R. J Biol Chem 2000;275:15799–808.
[61] Tomas-Zuber M, Mary JL, Lamour F, Bur D, Lesslauer W. J Biol Chem 2001;276:5892–9.
[62] Mackenzie SJ, Baille GS, McPhee I, Bolger GB, Houslay MD. J Biol Chem 2000;275:16609–17.
[63] Sharrocks AD. Nat Rev, Mol Cell Biol 2001;2:827–37.
[64] Elion EA. Curr Opin Microbiol 2000;3:573–81.
[65] Elion EA. J Cell Sci 2001;114:3967–78.
[66] Whitmarsh AJ, Cavanagh J, Tournier C, Yasuda J, Davis RJ. Science 1998;281:1671–4.
[67] Whitmarsh AJ, Davis RJ. Trends Biochem Sci 1998;23:481–5.
[68] Ito M, Yoshioka K, Akechi M, Yamashita S, Takamatsu N, Sugiyama K, et al. Mol Cell Biol 1999;19:7539–48.
[69] Yasuda J, Whitmarsh AJ, Cavanagh J, Sharma M, Davis RJ. Mol Cell Biol 1999;19:7245–54.
[70] Kelkar N, Gupta S, Dickens M, Davis RJ. Mol Cell Biol 2000; 20:1030–43.
[71] Whitmarsh AJ, Kuan CY, Kennedy NJ, Kelkar N, Haydar TF, Mordes JP, et al. Genes Dev 2002;15:2421–32.
[72] Bowman AB, Kamal A, Ritchings BW, Philp AV, McGrail M, Gindhart JG, et al. Cell 2000;103:583–94.
[73] Byrd DT, Kawasaki M, Walcoff M, Hisamoto N, Matsumoto K, Jin Y. Neuron 2001;32:787–800.
[74] Verhey KJ, Meyer D, Deehan R, Blenis J, Schnapp BJ, Rapoport TA, et al. J Cell Biol 2001;152:959–70.
[75] Meyer D, Liu A, Margolis BJ. Biol Chem 1999;274:35113–8.
[76] Stockinger W, Brandes C, Fasching D, Hermann M, Gotthardt M, Herz J, et al. Biol Chem 2000;275:25625–32.
[77] McDonald PH, Chow CW, Miller WE, Laporte SA, Field ME, Lin FT, et al. Science 2000;290:1574–7.
[78] Pitcher JA, Freedman NJ, Lefkowitz RJ. Ann Rev Biochem 1998; 67:653.
[79] Morrison DK. J Cell Sci 2001;114:1609–12.
[80] Therrien M, Chang HC, Solomon NM, Karim FD, Wassarman DA, Rubin GM. Cell 1995;83:879–88.
[81] Nguyen A, Burack WR, Stock JL, Kortum R, Chaika OV, Afkarian M, et al. Mol Cell Biol 2002;22:3035–45.
[82] Roy F, Laberge G, Douziech M, Ferland-McCollough D, Therrien M. Genes Dev 2002;16:427–38.
[83] Schaeffer HJ, Catling AD, Eblen ST, Collier LS, Krauss A, Weber MJ. Science 1998;281:1668–71.
[84] Xu S, Cobb MH. J Biol Chem 1997;272:32056–60.
[85] Karandikar M, Xu S, Cobb MH. J Biol Chem 2000;275:40120–7.

Available online at www.sciencedirect.com
SCIENCE DIRECT
Cellular Signalling 15 (2003) 463–469
CELLULAR SIGNALLING
www.elsevier.com/locate/cellsig

Review article

Mechanisms of regulating the Raf kinase family

Huira Chong[a], Haris G. Vikis[a], Kun-Liang Guan[a,b,*]

[a] *Department of Biological Chemistry, University of Michigan, Ann Arbor, MI 48109, USA*
[b] *The Institute of Gerontology, University of Michigan, Ann Arbor, MI 48109, USA*

Received 21 August 2002; accepted 9 October 2002

Abstract

The MAP Kinase pathway is a key signalling mechanism that regulates many cellular functions such as cell growth, transformation and apoptosis. One of the essential components of this pathway is the serine/threonine kinase, Raf. Raf (MAPKK kinase, MAPKKK) relays the extracellular signal from the receptor/Ras complex to a cascade of cytosolic kinases by phosphorylating and activating MAPK/ERK kinase (MEK; MAPK kinase, MAPKK) that phosphorylates and activates extracellular signal regulated kinase (ERK; mitogen-activated protein kinase, MAPK), which phosphorylates various cytoplasmic and nuclear proteins. Regulation of both Ras and Raf is crucial in the proper maintenance of cell growth as oncogenic mutations in these genes lead to high transforming activity. Ras is mutated in 30% of all human cancers and B-Raf is mutated in 60% of malignant melanomas. The mechanisms that regulate the small GTPase Ras as well as the downstream kinases MEK and extracellular signal regulated kinase (ERK) are well understood. However, the regulation of Raf is complex and involves the integration of other signalling pathways as well as intramolecular interactions, phosphorylation, dephosphorylation and protein–protein interactions. From studies using mammalian isoforms of Raf, as well as *C. elegans* lin45-Raf, common patterns and unique differences of regulation have emerged. This review will summarize recent findings on the regulation of Raf kinase.

Keywords: MAPK signalling; Ras; Phosphorylation; Scaffolding proteins

1. Introduction

Raf was originally identified as a retroviral oncogene (v-Raf) possessing serine/threonine kinase activity [1,2]. v-Raf-related genes were soon identified in humans and now comprise a family of three isoforms, A-Raf, B-Raf and C-Raf (Fig. 1). All Raf kinases are composed of three conserved regions, CR1, CR2 and CR3 [3]. The initial process of Raf activation involves the interaction of active GTP-bound Ras with the Ras binding domain (RBD) and cysteine rich domain (CRD) of CR1, and subsequent recruitment of Raf to the membrane for further activation [3–5]. The role of the serine/threonine-rich CR2 is less defined; however, phosphorylation of CR2 and various protein–protein interactions via CR2 appear to affect the localization and activation of Raf [6–10]. Deletions of the N-terminal regulatory domains (CR1 and CR2) similar to v-Raf are found in several activated forms of Raf genes detected in certain neoplastic human cells, which suggests that these domains negatively regulate Raf [11–14]. CR3 is the catalytic kinase domain of Raf and is also subject to regulation by phosphorylation. Hence, the regulation of Raf kinases is a complex process involving inter- and intramolecular interactions as well as phosphorylation of the regulatory and catalytic domains of the protein [3,9,10,15–17].

Studies with Raf knockout mice have revealed both overlapping and unique functions for Raf isoforms [18–23]. For example, in C-Raf knockout mice, B-Raf can compensate for C-Raf in the activation of MAPK/ERK kinase (MEK). However, these mice are more susceptible to apoptotic stimuli despite the presence of A- and B-Raf [20]. Biochemical differences also exist between Raf iso-

Abbreviations: CNK, connector enhancer of KSR; CRD, cysteine rich domain; ERK, extracellular signal regulated kinase; JAK, Janus activated kinase; KSR, kinase suppressor of Ras; MAGUIN, membrane-associated guanylate kinase-interacting protein; MAPK, mitogen activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPKK kinase; MEK, MAPK/ERK kinase; PAK, p21 activated kinase; PKA, protein kinase A; PKC, protein kinase C; RBD, Ras binding domain; RKIP, Raf kinase inhibitor protein; SGK, serum and glucocorticoid activated kinase.

* Corresponding author. Department of Biological Chemistry, University of Michigan, Ann Arbor, MI 48109, USA. Tel.: +1-734-763-3060; fax: +1-734-763-4581.
E-mail address: kunliang@umich.edu (K.-L. Guan).

0898-6568/02/$ - see front matter © 2002 Elsevier Science Inc. All rights reserved.
doi:10.1016/S0898-6568(02)00139-0

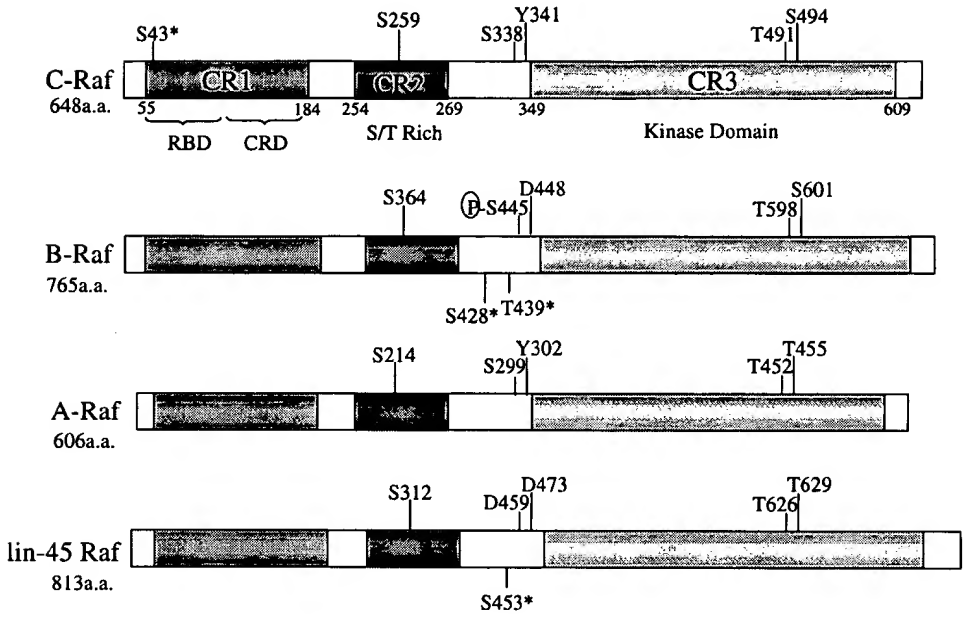

Fig. 1. The conserved structure of the Raf kinase family. The three conserved regions of Raf kinases are conserved across isoforms and species. CR1 has two Ras binding domains (RBD and CRD), CR2 is serine/threonine rich and CR3 is the catalytic kinase domain. Conserved regulatory phosphorylation sites are also shown. Significant regulatory sites that are not conserved but are acidic residues in other isoforms/species are noted. Other phosphorylation sites that are not conserved are marked with an asterisk (*). S43 and S259 in C-Raf, S364, S428 and T439 in B-Raf and S312 and S453 in lin-45 Raf are inhibitory phosphorylation sites. All other regulatory sites noted are activating phosphorylation sites.

forms. A-Raf is a much weaker activator of MEK compared to B- and C-Raf. Furthermore, A-Raf can only activate MEK1 where C-Raf is able to activate both MEK1 and MEK2 [24–26]. Although highly conserved at the primary sequences, Raf isoforms have similar and distinct biochemical and functional properties.

Raf isoforms also vary in their cell-specific expression and subcellular localization. For example, B-Raf (present as alternatively spliced isoforms) and C-Raf (expressed in many tissues) are expressed in the brain, while A-Raf is not [27]. A- and C-Raf are highly expressed in muscle, whereas B-Raf is detectable only at very low levels. In

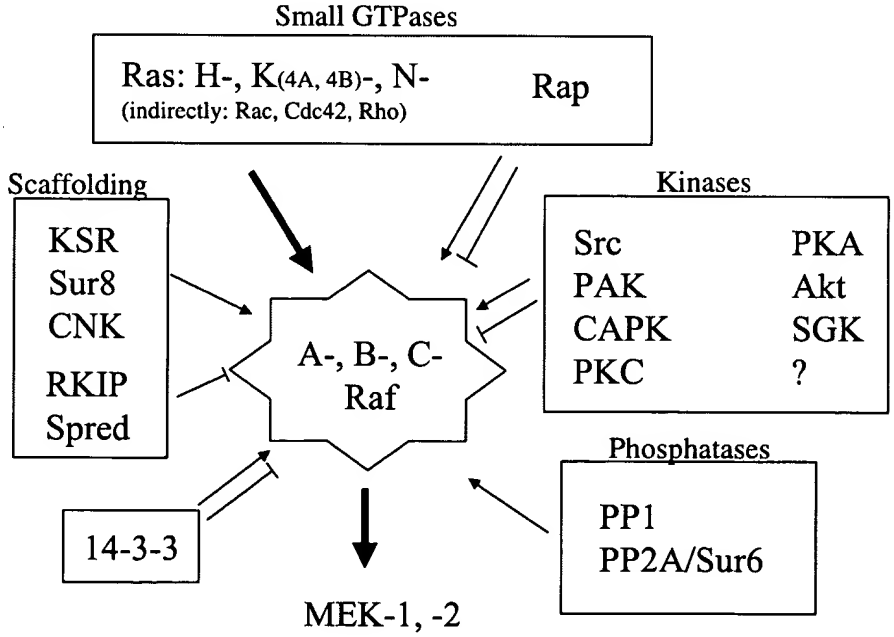

Fig. 2. Raf is a point of regulation in the MAP kinase pathway. Small GTPases, kinases, phosphatases, scaffolding proteins and 14-3-3 regulate the MAP kinase pathway through their effects on Raf kinase activity.

addition, Raf kinases are also localized to different subcellular compartments. Both A- and C-Raf have stable or transient localization to the mitochondria, which has implicated Raf in the regulation of apoptosis [28–31]. B- and C-Raf are both expressed in neurons; however, B-Raf is localized to neurite processes and C-Raf is perinuclear [27]. This localization may be due to isoform-specific lipid or protein-binding partners, which recruit Raf to distinct membrane rafts.

All Raf kinases are activated by the Ras small GTPase, regulatory phosphorylation events and scaffolding proteins (Fig. 2). The combination of all these events leads to the proper activation of Raf. Hence, the complexity of Raf activation lies in the fact that different Raf isoforms combine common and unique mechanisms to regulate Raf kinase activity.

2. Activation by small GTPases

Recruitment of Raf to the plasma membrane by the small GTPase Ras is the initial event in the Raf activation process. The effector domain of GTP-bound Ras binds to Raf through two domains in CR1, the Ras-binding domain (RBD) and the cysteine-rich domain (CRD) [3–5]. Binding to both sites is necessary for Raf activation. Although all Ras isoforms bind Raf with comparable affinity, different Ras isoforms activate Raf kinases to varying degrees. For example, K-Ras is a more potent activator of C-Raf than is H-Ras [32]. In addition, Raf activation by Ras is also affected by the subcellular localization of the various Ras isoforms through their prenylation signals, which function to target them to membranes of specific lipid content [33–35]. It has been recently shown that the prenylation signal on H- and N-Ras can localize these molecules to the ER and Golgi membrane, respectively [34]. Localization of Ras to these organelle membranes was shown to affect mitogen activated protein kinase (MAPK) signalling with kinetics different from signalling from the plasma membrane [34].

The small GTPase Rap has an identical effector domain to that of Ras, but whose activation results in different downstream functions [36]. Notably, Rap activates B-Raf but inhibits C-Raf activation. Rap binds the CRD of C-Raf with higher affinity than Ras [37] and excludes Ras from binding. Replacement of the CRD in C-Raf with the CRD from B-Raf results in the loss of Rap inhibition of C-Raf. Similarly, replacement of the CRD in B-Raf with the CRD from C-Raf results in the inhibition of B-Raf by Rap. The mechanism of Rap inhibition of C-Raf further suggests that small GTPase binding alone is insufficient for C-Raf activation.

The Rho family of small GTPases has been implicated in C-Raf activation. The Rho family, which consists of Rho, Rac and Cdc42, regulates cytoskeletal structures such as stress fibres, lamellipodia and filopodia, respectively. However, these GTPases do not directly bind Raf but signal via activation of downstream kinases. For example, Rho can signal through the activation of PKN1, PRK2 and ROCK1, where Rac and Cdc42 can signal through p21 activated kinase (PAK). Studies of C-Raf activation have led to the identification of a site phosphorylated by PAK (S338) that is critical to C-Raf activity [38–41]. Consistent with the role of PAK, the upstream activators Rac and Cdc42 cooperate with Ras to activate C-Raf [42]. One study showed that by using activated Ras mutants with an additional mutation that impairs C-Raf activation, but not Raf binding, activated Rac and Cdc42 were able to rescue C-Raf activation. However, activated Rac and Cdc42 alone could not induce C-Raf activity, and membrane localization of C-Raf was still required for activation. A dominant negative Rac mutant was able to abrogate Ras induction of C-Raf activity, which suggests that endogenous Rac is required for C-Raf activation. Similar observations were made with activated Rho; however, the kinase-activated downstream of Rho is unknown. Hence, it appears that the members of the Rho family of small GTPases are critical mediators which contribute indirectly to Ras-induced activation of Raf.

3. Phosphorylation

Phosphorylation is an important mechanism by which Raf activity is regulated. Most studies on Raf regulation have focused on C-Raf, but studies involving B-Raf, C-Raf and the *C. elegans* lin45-Raf have revealed that there is a conserved mechanism of regulation involving activation and inhibition by phosphorylation and dephosphorylation. Although the Raf kinases are structurally conserved between isoforms and species, there exist unique differences at key individual phosphorylation sites. Such differences explain how B- and C-Raf are both activated by Ras but can be regulated differently.

Ras activation induces phosphorylation of Raf at numerous residues; however, a number of residues are also phosphorylated in the basal, unstimulated state. These residues include S259 and S621 which were originally identified in C-Raf [7]. Phosphorylation of S259 and S621 creates two 14-3-3 binding sites which presumably help keep C-Raf in an auto-inhibited state. Phosphorylation at S621 may have more complex implications since mutation of this residue leads to complete inactivation of the kinase. Hence, a balance of phosphorylation and dephosphorylation is required to prime Raf properly in the basal state prior to stimulation by Ras or mitogens.

C-Raf has four activating phosphorylation sites that are Ras-inducible: S338, Y341, T491 and S494. S338 is the PAK phosphorylation site which lies just N-terminal to the kinase domain and is critical for Raf activation [15,38–43]. This site is conserved in the mammalian Rafs, as well as in *Drosophila* D-Raf. Interestingly, the homologous site in B-Raf, S445, is constitutively phosphorylated and may account for the high basal activity of B-Raf compared to

C-Raf [41]. *C. elegans* lin45-Raf has an aspartic acid residue substituted at this position which may mimic the effect of phosphorylation. Mutation of S338 in C-Raf results in a reduction of Raf activation induced by EGF, activated Ras, phorbol esters and the muscarinic G-protein coupled receptor pathway. Ras-induced phosphorylation of this site presumably occurs through Ras activation of PI3 kinase, whose lipid byproducts indirectly activate Rac exchange factors. Activated mutants of Rac and Cdc42 were also able to induce phosphorylation at S338, through activation of PAK.

Another C-Raf phosphorylation site is Y341 [24,41,44–47], which also lies just N-terminal to the kinase domain. Y341 is phosphorylated by Src and Janus activated kinase (JAK) [44,48,49]. In B-Raf, this tyrosine is replaced by an aspartic acid residue which can explain why B-Raf can be fully induced by Ras alone, but A- and C-Raf also require Src for full activation. A mutation in C-Raf that no longer binds Ras and therefore cannot be recruited to the membrane is also compromised in activation by Src [41,46]. Raf activation can be rescued if the mutant is artificially targeted to the plasma membrane where it can be activated by Src alone. However, Ras-mediated membrane recruitment and Src activation are not the only steps in C-Raf activation.

Two phosphorylation sites, T491 and S494, have been identified in the activation loop of the kinase domain of C-Raf [15]. These sites were first identified in B-Raf (T598 and S601), where mutation to alanine residues resulted in a loss of B-Raf activity induced by EGF and activated Ras, as well as by phorbol esters and muscarinic G-protein-coupled receptors [9]. Mutation of these sites in B-Raf to phosphomimetic residues resulted in constitutive activity independent of activated Ras. Recently, V599 in B-Raf was found to be frequently mutated to glutamic acid in human malignant melanomas and to a lower frequency in other cancers [50]. This tumour-associated mutation creates a negative charge next to the T598 phosphorylation site in the activation loop and is sufficient to activate B-Raf activity. The activation loop phosphorylation sites are also conserved across species. Acidic residue substitutions at homologous sites in lin45-Raf result in a multi-vulval phenotype indicative of an activated MAPK pathway [15]. The same acidic residue substitutions in C-Raf result in higher basal activity; however, Raf activity can be further enhanced by Ras through phosphorylation of S338 and Y341. T491 and S494 are phosphorylated in a Ras-dependent manner; however, the kinase that phosphorylates these residues has not been identified. Substitution of all four activating phosphorylation sites, S338, Y341, T491 and S494, to acidic residues results in full C-Raf kinase activity.

Phorbol esters, which activate protein kinase C (PKC), can activate Raf kinase activity. S499, which lies in the activation loop of C-Raf, was originally identified as a site phosphorylated by PKC [51]. However, several studies have found that mutation of S499 to alanine has no effect on PKC-stimulated Raf activity or Raf activity induced by activated Ras or EGF [15,52]. Hence, the role of phosphorylation of S499 as well as the mechanism of PKC-stimulated Raf activity still remains to be elucidated. A similar observation has occurred for S43 (located in the RBD) which was one of the first C-Raf phosphorylation sites identified [7]. S43 in C-Raf was thought to be directly phosphorylated by protein kinase A (PKA) and mediate PKA-induced inhibition [53]. However, it was shown that mutation of this site had no effect on PKA-induced C-Raf inhibition [54]. In B-Raf, this site is not conserved but B-Raf is still inhibited by forskolin-induced PKA [54,55]. Adding to the complexity of PKA-regulated Raf activity is the fact that PKA also stimulates Rap, which activates B-Raf [56]. The effect of PKA on C-Raf has been found to vary depending on cell type and serum conditions, which has led to confusion about the role of PKA and S43 in C-Raf regulation. This was clarified by the observation that during serum starvation, phosphorylation at S43 inhibits Ras binding to the RBD, and therefore inhibits C-Raf activation [57]. However, in the presence of mitogenic factors, C-Raf phosphorylated on S43 is able to bind Ras and thus this site plays no role in regulating C-Raf activity. The same study also showed that PKA can phosphorylate S43 as well as S259.

Inhibition by phosphorylation is a conserved mechanism of Raf regulation. In addition to S43, C-Raf contains an inhibitory phosphorylation site at S259. The current model suggests that phosphorylation of S259 along with phosphorylation of S621 creates an auto-inhibited conformation state that is maintained by a 14-3-3 dimer, thus bridging the N- and C-terminal domains together [3]. Akt was identified as the kinase that phosphorylates S259 and thus inhibits Raf activity [6,10,58]. Mutation of this site to alanine increases the basal activity of C-Raf. S259 is also conserved among the Raf isoforms as well as in *C. elegans* and *Drosophila* Raf kinases. However, in addition to the conserved S259 residue, B-Raf contains two additional Akt sites, S428 and T439. Unlike C-Raf, mutation of C-Raf S259 site in B-Raf (S364) to alanine results in a minor increase in basal activity [6,15]. Only when all three sites are mutated to alanine does the activity increase to a level comparable to Ras-stimulated activity. Similarly, single mutation of either of the two putative Akt sites to alanine in *C. elegans* lin-45 Raf has no phenotypic effect; however, mutation of both sites results in a mutli-vulval phenotype [15]. S364 in B-Raf has also been identified as a serum and glucocorticoid-inducible kinase (SGK) phosphorylation site [59]. SGK has some homology to Akt but, unlike Akt, preferentially targets S364 on B-Raf. Whether or not SGK regulation is unique to B-Raf or common to all Raf isoforms remains to be examined.

The serine/threonine phosphatases PP1 and PP2A have been shown to promote C-Raf activation [60–62]. The positive role of these phosphatases in the regulation of Raf activity was demonstrated initially in *C. elegans* [61]. A genetic screen identified Sur6, a subunit of PP2A, as an activator of the MAPK pathway. It was observed that the multi-vulval phenotype induced by activated Ras was

reduced by a mutation in Sur6, which suggests that Sur6 plays a positive role in signalling. These data also propose that the phosphatase acts downstream of Ras in the pathway. A study by Jaumot and Hancock [62] found that the critical target of PP1 and PP2A is S259. Mutation of S259 to alanine resulted in high basal activity. General and specific inhibitors of the PP1 and PP2A phosphatases abrogated Ras-stimulated C-Raf activity. Furthermore, inhibition of PP1 and PP2A resulted in a shift in C-Raf membrane microdomain localization and also in the increase of 14-3-3/C-Raf complexes at the plasma membrane. These complexes were not able to be activated even though they were localized to the plasma membrane. These data further support that membrane recruitment alone is insufficient for Raf activation and that PP1 and PP2A regulate microdomain localization of Raf which is important for Raf activity [62].

4. Scaffolding proteins

Genetic screens in *Drosophila* and *C. elegans* have revealed several novel genes that are important modulators of the Ras/MAPK pathway. One of these genes, called kinase suppressor of Ras (KSR), functions as a positive regulator and is proposed to act downstream of or in parallel to Ras [63–65]. KSR contains a C-terminal kinase domain with some homology to the Raf kinase family. However, the KSR kinase domain contains an arginine residue in place of a conserved catalytic lysine residue. Nevertheless, an initial report concluded that KSR does in fact possess kinase activity, is activated by ceramide and results in the direct phosphorylation of T269 of C-Raf to enhance activity [66]. Further observations showed that EGF can stimulate KSR activity (via an unknown mechanism) and that KSR mediates optimal Raf activation through phosphorylation of T269 [67,68]. However, several groups have been unable to detect any catalytic function for KSR and have proposed that KSR functions as a scaffolding protein based on its ability to interact with Raf, MEK, ERK, 14-3-3 and various heat shock proteins [69–72]. Expression of KSR in mammalian cells is able to redistribute MEK from the soluble fraction into a high molecular weight membrane-associated complex, suggestive of a function as a scaffolding protein [71]. A recent report suggests that KSR functions as a scaffold to facilitate the phosphorylation of MEK by Raf [73].

Observations made in *C. elegans* suggested that KSR kinase activity is not necessary for KSR function [71]. Predicted kinase-dead mutants of KSR were able to complement a KSR loss-of-function allele. However, recent analysis of the *C. elegans* genome has uncovered a second KSR gene, ksr-2 [74]. It was observed that only double mutants of ksr-1 and ksr-2 had strong defects in vulval development. This suggested that ksr-1 and ksr-2 are functionally redundant; however, both are essential for MAPK signalling in *C. elegans*. Ablation of the KSR gene in mouse resulted in a phenotypically normal animal in contrast to observations made in *C. elegans* [75]. However, the mouse did exhibit attenuated ERK activity and loss of the previously described high molecular weight scaffolding complex. In *Drosophila* S2 cells, reduction of KSR protein levels using RNAi impaired insulin-stimulated Raf activation [76]. Together, these studies suggest that although KSR may not be an essential component of MAPK signalling in mammals, it is required for enhanced MAPK signalling, hence supporting its role as a modulator.

Another product isolated from genetic screens is Sur8/Soc-2 [77,78]. Epistatic experiments have predicted Sur-8 to function downstream of Ras and upstream of Raf. Analysis of the Sur-8 sequence revealed multiple leucine-rich repeats which suggest its involvement in protein–protein interactions. Human Sur-8 is able to enhance Ras and EGF-stimulated Raf activity, but has no effect on Raf- or MEK-induced ERK activity [79]. Sur-8 is able to form a ternary complex with Ras and Raf, which suggests that its function is to facilitate activation by bringing these molecules into close proximity.

A third protein identified through genetic screens is connector enhancer of KSR (CNK). Like KSR and Sur-8, epistatic studies in *Drosophila* place CNK downstream of Ras and parallel or upstream of Raf [80]. Using double-stranded RNA to knockdown expression, Anselmo et al. [76] showed that CNK and KSR in *Drosophila* S2 cells are required for Raf activation and that CNK specifically enhances the membrane recruitment of Raf. CNK has no apparent catalytic domain, but has multiple protein–protein interaction domains and localizes to cell–cell contact regions. CNK may be able to associate with other Ras-mediated, MAPK-independent pathways such as the RalGDS pathway [81]. The role of the mammalian CNK homologue, membrane-associated guanylate kinase-interacting protein (MAGUIN)-1, in Raf activation is still elusive [82].

The scaffolding proteins mentioned above enhance MAPK signalling. Raf kinase inhibitor protein (RKIP) is a scaffolding protein that inhibits the MAPK pathway [83]. Raf kinase inhibitor protein (RKIP) can bind Raf, MEK and ERK; however, the binding of Raf and MEK to RKIP is mutually exclusive [84]. The Raf and MEK binding sites on RKIP overlap and exclude each other from binding by steric interference. Suppression of endogenous RKIP expression was found to induce MEK/ERK activity. RKIP functions by inhibiting the formation of Raf and MEK in the same signalling module regardless of the activation state of Raf. Whether RKIP is specific to C-Raf or whether other isoforms have their own RKIP-like scaffolding inhibitor is unknown.

An even more potent inhibitor of the MAP kinase pathway is the Sprouty-related protein, Spred [85]. Sprouty was first identified in a genetic screen in *Drosophila* and was found to be a general inhibitor of receptor tyrosine kinases

[86,87]. Subsequently, Sprouty was shown to inhibit the formation of functional signalling complexes with the receptor and upstream of Ras [88]. Unlike Sprouty, Spred inhibits the MAP kinase pathway downstream of Ras and is able to associate with Raf. Although Spred binds Ras, Spred does not affect the activation of Ras or the recruitment of Raf to the membrane. However, it does inhibit Raf phosphorylation and activation by as yet undetermined mechanism [85]. The presence of enhancing and inhibitory scaffolding proteins adds another dimension to the diversity of Raf regulation.

5. Concluding remarks

The activation of Raf involves a complex series of events that include membrane recruitment, phosphorylation and dephosphorylation and the disruption and formation of various protein–protein interactions. Recent work has elucidated several new phosphorylation sites that are critical to the activation of Raf and also the signalling pathways that influence the phosphorylation state of Raf. In addition, genetic experiments in *Drosophila* and *C. elegans* have uncovered numerous Raf-binding proteins that scaffold the Raf complex and modulate its activation, which has added to the sheer complexity of the activation process. What has also become clear in recent years is that Raf isoforms share both common and unique mechanisms by which they are regulated. Since Raf isoforms have been shown to be expressed in different tissues and within various subcellular compartments, this suggests that it is not necessarily accurate to apply canonical mechanisms of Raf activation and function to each isoform. The complexity and diversity of Raf regulation continue to grow due to concerted efforts in the fields of genetics, biochemistry and cell biology. Future work in this area will undoubtedly take a more detailed analysis based on an isoform-, cell type- and stimulus-specific context of Raf signalling.

Acknowledgements

We would like to thank the members of the Guan Laboratory for helpful discussions. The authors are supported by the NIH National Research Service Award #5-T32-GM07544 (HC), the NIH, the Walthier Cancer Institute and a John D. and Katherine T. MacArthur Fellowship (KLG).

References

[1] Moelling K, Heimann B, Beimling P, Rapp UR, Sander T. Nature 1984;312(5994):558–61.
[2] Rapp UR, Goldsborough MD, Mark GE, Bonner TI, Groffen J, Reynolds Jr FH, et al. Proc Natl Acad Sci U S A 1983;80(14):4218–22.
[3] Morrison DK, Cutler RE. Curr Opin Cell Biol 1997;9(2):174–9.
[4] Nassar N, Horn G, Herrmann C, Scherer A, McCormick F, Wittinghofer A. Nature 1995;375(6532):554–60.
[5] Vojtek AB, Hollenberg SM, Cooper JA. Cell 1993;74(1):205–14.
[6] Guan KL, Figueroa C, Brtva TR, Zhu T, Taylor J, Barber TD, et al. J Biol Chem 2000;275(35):27354–9.
[7] Morrison DK, Heidecker G, Rapp UR, Copeland TD. J Biol Chem 1993;268(23):17309–16.
[8] Yao B, Zhang Y, Delikat S, Mathias S, Basu S, Kolesnick R. Nature 1995;378(6554):307–10.
[9] Zhang BH, Guan KL. EMBO J 2000;19(20):5429–39.
[10] Zimmermann S, Moelling K. Science 1999;286(5445):1741–4.
[11] Fukui M, Yamamoto T, Kawai S, Maruo K, Toyoshima K. Proc Natl Acad Sci U S A 1985;82(17):5954–8.
[12] Kasid U, Pfeifer A, Weichselbaum RR, Dritschilo A, Mark GE. Science 1987;237(4818):1039–41.
[13] Shimizu K, Nakatsu Y, Sekiguchi M, Hokamura K, Tanaka K, Terada M, et al. Proc Natl Acad Sci U S A 1985;82(17):5641–5.
[14] Stanton Jr VP, Cooper GM. Mol Cell Biol 1987;7(3):1171–9.
[15] Chong H, Lee J, Guan KL. EMBO J 2001;20(14):3716–27.
[16] Cutler Jr RE, Stephens RM, Saracino MR, Morrison DK. Proc Natl Acad Sci U S A 1998;95(16):9214–9.
[17] Heidecker G, Huleihel M, Cleveland JL, Kolch W, Beck TW, Lloyd P, et al. Mol Cell Biol 1990;10(6):2503–12.
[18] Huser M, Luckett J, Chiloeches A, Mercer K, Iwobi M, Giblett S, et al. EMBO J 2001;20(8):1940–51.
[19] Mercer K, Chiloeches A, Huser M, Kiernan M, Marais R, Pritchard C. Oncogene 2002;21(3):347–55.
[20] Mikula M, Schreiber M, Husak Z, Kucerova L, Ruth J, Wieser R, et al. EMBO J 2001;20(8):1952–62.
[21] Pritchard CA, Bolin L, Slattery R, Murray R, McMahon M. Curr Biol 1996;6(5):614–7.
[22] Wojnowski L, Zimmer AM, Beck TW, Hahn H, Bernal R, Rapp UR, et al. Nat Genet 1997;16(3):293–7.
[23] Wojnowski L, Stancato LF, Larner AC, Rapp UR, Zimmer A. Mech Dev 2000;91(1–2):97–104.
[24] Marais R, Light Y, Paterson HF, Mason CS, Marshall CJ. J Biol Chem 1997;272(7):4378–83.
[25] Pritchard CA, Samuels ML, Bosch E, McMahon M. Mol Cell Biol 1995;15(11):6430–42.
[26] Wu X, Noh SJ, Zhou G, Dixon JE, Guan KL. J Biol Chem 1996;271(6):3265–71.
[27] Morice C, Nothias F, Konig S, Vernier P, Baccarini M, Vincent JD, et al. Eur J Neurosci 1999;11(6):1995–2006.
[28] Majewski M, Nieborowska-Skorska M, Salomoni P, Slupianek A, Reiss K, Trotta R, et al. Cancer Res 1999;59(12):2815–9.
[29] Nantel A, Huber M, Thomas DY. J Biol Chem 1999;274(50):35719–24.
[30] Wang HG, Rapp UR, Reed JC. Cell 1996;87(4):629–38.
[31] Yuryev A, Ono M, Goff SA, Macaluso F, Wennogle LP. Mol Cell Biol 2000;20(13):4870–8.
[32] Yan J, Roy S, Apolloni A, Lane A, Hancock JF. J Biol Chem 1998;273(37):24052–6.
[33] Apolloni A, Prior IA, Lindsay M, Parton RG, Hancock JF. Mol Cell Biol 2000;20(7):2475–87.
[34] Chiu VK, Bivona T, Hach A, Sajous JB, Silletti J, Wiener H, et al. Nat Cell Biol 2002;4(5):343–50.
[35] Choy E, Chiu VK, Silletti J, Feoktistov M, Morimoto T, Michaelson D, et al. Cell 1999;98(1):69–80.
[36] Ohtsuka T, Shimizu K, Yamamori B, Kuroda S, Takai Y. J Biol Chem 1996;271(3):1258–61.
[37] Okada T, Hu CD, Jin TG, Kariya K, Yamawaki-Kataoka Y, Kataoka T. Mol Cell Biol 1999;19(9):6057–64.
[38] Chaudhary A, King WG, Mattaliano MD, Frost JA, Diaz B, Morrison DK, et al. Curr Biol 2000;10(9):551–4.

[39] Diaz B, Barnard D, Filson A, MacDonald S, King A, Marshall M. Mol Cell Biol 1997;17(8):4509–16.
[40] King AJ, Sun H, Diaz B, Barnard D, Miao W, Bagrodia S, et al. Nature 1998;396(6707):180–3.
[41] Mason CS, Springer CJ, Cooper RG, Superti-Furga G, Marshall CJ, Marais R. EMBO J 1999;18(8):2137–48.
[42] Li W, Chong H, Guan KL. J Biol Chem 2001;276(37):34728–37.
[43] Sun H, King AJ, Diaz HB, Marshall MS. Curr Biol 2000;10(5): 281–4.
[44] Fabian JR, Daar IO, Morrison DK. Mol Cell Biol 1993;13(11):7170–9.
[45] Jelinek T, Dent P, Sturgill TW, Weber MJ. Mol Cell Biol 1996;16(3): 1027–34.
[46] Marais R, Light Y, Paterson HF, Marshall CJ. EMBO J 1995;14(13): 3136–45.
[47] Stokoe D, McCormick F. EMBO J 1997;16(9):2384–96.
[48] Winston LA, Hunter T. J Biol Chem 1995;270(52):30837–40.
[49] Xia K, Mukhopadhyay NK, Inhorn RC, Barber DL, Rose PE, Lee RS, et al. Proc Natl Acad Sci U S A 1996;93(21):11681–6.
[50] Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, et al. Nature 2002;417(6892):949–54.
[51] Kolch W, Heidecker G, Kochs G, Hummel R, Vahidi H, Mischak H, et al. Nature 1993;364(6434):249–52.
[52] Barnard D, Diaz B, Clawson D, Marshall M. Oncogene 1998; 17(12):1539–47.
[53] Schramm K, Niehof M, Radziwill G, Rommel C, Moelling K. Biochem Biophys Res Commun 1994;201(2):740–7.
[54] Sidovar MF, Kozlowski P, Lee JW, Collins MA, He Y, Graves LM. J Biol Chem 2000;275(37):28688–94.
[55] Konig S, Guibert B, Morice C, Vernier P, Barnier JV. C R Acad Sci, Ser III 2001;324(8):673–81.
[56] Quilliam LA, Mueller H, Bohl BP, Prossnitz V, Sklar LA, Der CJ, et al. J Immunol 1991;147(5):1628–35.
[57] Dhillon AS, Pollock C, Steen H, Shaw PE, Mischak H, Kolch W. Mol Cell Biol 2002;22(10):3237–46.
[58] Rommel C, Clarke BA, Zimmermann S, Nunez L, Rossman R, Reid K, et al. Science 1999;286(5445):1738–41.
[59] Zhang BH, Tang ED, Zhu T, Greenberg ME, Vojtek AB, Guan KL. J Biol Chem 2001;276(34):31620–6.
[60] Abraham D, Podar K, Pacher M, Kubicek M, Welzel N, Hemmings BA, et al. J Biol Chem 2000;275(29):22300–4.
[61] Sieburth DS, Sundaram M, Howard RM, Han M. Genes Dev 1999; 13(19):2562–9.
[62] Jaumot M, Hancock JF. Oncogene 2001;20(30):3949–58.
[63] Kornfeld K, Hom DB, Horvitz HR. Cell 1995;83(6):903–13.
[64] Sundaram M, Han M. Cell 1995;83(6):889–901.
[65] Therrien M, Chang HC, Solomon NM, Karim FD, Wassarman DA, Rubin GM. Cell 1995;83(6):879–88.
[66] Zhang Y, Yao B, Delikat S, Bayoumy S, Lin XH, Basu S, et al. Cell 1997;89(1):63–72.
[67] Xing HR, Kolesnick R. J Biol Chem 2001;276(13):9733–41.
[68] Xing HR, Lozano J, Kolesnick R. J Biol Chem 2000;275(23): 17276–80.
[69] Cacace AM, Michaud NR, Therrien M, Mathes K, Copeland T, Rubin GM, et al. Mol Cell Biol 1999;19(1):229–40.
[70] Morrison DK. J Cell Sci 2001;114(Pt 9):1609–12.
[71] Stewart S, Sundaram M, Zhang Y, Lee J, Han M, Guan KL. Mol Cell Biol 1999;19(8):5523–34.
[72] Yu W, Fantl WJ, Harrowe G, Williams LT. Curr Biol 1998;8(1):56–64.
[73] Roy F, Laberge G, Douziech M, Ferland-McCollough D, Therrien M. Genes Dev 2002;16(4):427–38.
[74] Ohmachi M, Rocheleau CE, Church D, Lambie E, Schedl T, Sundaram MV. Curr Biol 2002;12(5):427–33.
[75] Nguyen A, Burack WR, Stock JL, Kortum R, Chaika OV, Afkarian M, et al. Mol Cell Biol 2002;22(9):3035–45.
[76] Anselmo AN, Bumeister R, Thomas JM, White MA. J Biol Chem 2002;277(8):5940–3.
[77] Selfors LM, Schutzman JL, Borland CZ, Stern MJ. Proc Natl Acad Sci U S A 1998;95(12):6903–8.
[78] Sieburth DS, Sun Q, Han M. Cell 1998;94(1):119–30.
[79] Li W, Han M, Guan KL. Genes Dev 2000;14(8):895–900.
[80] Therrien M, Wong AM, Rubin GM. Cell 1998;95(3):343–53.
[81] Therrien M, Wong AM, Kwan E, Rubin GM. Proc Natl Acad Sci U S A 1999;96(23):13259–63.
[82] Yao I, Ohtsuka T, Kawabe H, Matsuura Y, Takai Y, Hata Y. Biochem Biophys Res Commun 2000;270(2):538–42.
[83] Yeung K, Seitz T, Li S, Janosch P, McFerran B, Kaiser C, et al. Nature 1999;401(6749):173–7.
[84] Yeung K, Janosch P, McFerran B, Rose DW, Mischak H, Sedivy JM, et al. Mol Cell Biol 2000;20(9):3079–85.
[85] Wakioka T, Sasaki A, Kato R, Shouda T, Matsumoto A, Miyoshi K, et al. Nature 2001;412(6847):647–51.
[86] Hacohen N, Kramer S, Sutherland D, Hiromi Y, Krasnow MA. Cell 1998;92(2):253–63.
[87] Reich A, Sapir A, Shilo B. Development 1999;126(18):4139–47.
[88] Casci T, Vinos J, Freeman M. Cell 1999;96(5):655–65.

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Cellular Signalling 15 (2003) 463–469
CELLULAR SIGNALLING
www.elsevier.com/locate/cellsig

Review article

Mechanisms of regulating the Raf kinase family

Huira Chong[a], Haris G. Vikis[a], Kun-Liang Guan[a,b,*]

[a] *Department of Biological Chemistry, University of Michigan, Ann Arbor, MI 48109, USA*
[b] *The Institute of Gerontology, University of Michigan, Ann Arbor, MI 48109, USA*

Received 21 August 2002; accepted 9 October 2002

Abstract

The MAP Kinase pathway is a key signalling mechanism that regulates many cellular functions such as cell growth, transformation and apoptosis. One of the essential components of this pathway is the serine/threonine kinase, Raf. Raf (MAPKK kinase, MAPKKK) relays the extracellular signal from the receptor/Ras complex to a cascade of cytosolic kinases by phosphorylating and activating MAPK/ERK kinase (MEK; MAPK kinase, MAPKK) that phosphorylates and activates extracellular signal regulated kinase (ERK; mitogen-activated protein kinase, MAPK), which phosphorylates various cytoplasmic and nuclear proteins. Regulation of both Ras and Raf is crucial in the proper maintenance of cell growth as oncogenic mutations in these genes lead to high transforming activity. Ras is mutated in 30% of all human cancers and B-Raf is mutated in 60% of malignant melanomas. The mechanisms that regulate the small GTPase Ras as well as the downstream kinases MEK and extracellular signal regulated kinase (ERK) are well understood. However, the regulation of Raf is complex and involves the integration of other signalling pathways as well as intramolecular interactions, phosphorylation, dephosphorylation and protein–protein interactions. From studies using mammalian isoforms of Raf, as well as *C. elegans* lin45-Raf, common patterns and unique differences of regulation have emerged. This review will summarize recent findings on the regulation of Raf kinase.

Keywords: MAPK signalling; Ras; Phosphorylation; Scaffolding proteins

1. Introduction

Raf was originally identified as a retroviral oncogene (v-Raf) possessing serine/threonine kinase activity [1,2]. v-Raf-related genes were soon identified in humans and now comprise a family of three isoforms, A-Raf, B-Raf and C-Raf (Fig. 1). All Raf kinases are composed of three conserved regions, CR1, CR2 and CR3 [3]. The initial process of Raf activation involves the interaction of active GTP-bound Ras with the Ras binding domain (RBD) and cysteine rich domain (CRD) of CR1, and subsequent recruitment of Raf to the membrane for further activation [3–5]. The role of the serine/threonine-rich CR2 is less defined; however, phosphorylation of CR2 and various protein–protein interactions via CR2 appear to affect the localization and activation of Raf [6–10]. Deletions of the N-terminal regulatory domains (CR1 and CR2) similar to v-Raf are found in several activated forms of Raf genes detected in certain neoplastic human cells, which suggests that these domains negatively regulate Raf [11–14]. CR3 is the catalytic kinase domain of Raf and is also subject to regulation by phosphorylation. Hence, the regulation of Raf kinases is a complex process involving inter- and intramolecular interactions as well as phosphorylation of the regulatory and catalytic domains of the protein [3,9,10,15–17].

Studies with Raf knockout mice have revealed both overlapping and unique functions for Raf isoforms [18–23]. For example, in C-Raf knockout mice, B-Raf can compensate for C-Raf in the activation of MAPK/ERK kinase (MEK). However, these mice are more susceptible to apoptotic stimuli despite the presence of A- and B-Raf [20]. Biochemical differences also exist between Raf iso-

Abbreviations: CNK, connector enhancer of KSR; CRD, cysteine rich domain; ERK, extracellular signal regulated kinase; JAK, Janus activated kinase; KSR, kinase suppressor of Ras; MAGUIN, membrane-associated guanylate kinase-interacting protein; MAPK, mitogen activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPKK kinase; MEK, MAPK/ERK kinase; PAK, p21 activated kinase; PKA, protein kinase A; PKC, protein kinase C; RBD, Ras binding domain; RKIP, Raf kinase inhibitor protein; SGK, serum and glucocorticoid activated kinase.

* Corresponding author. Department of Biological Chemistry, University of Michigan, Ann Arbor, MI 48109, USA. Tel.: +1-734-763-3060; fax: +1-734-763-4581.
E-mail address: kunliang@umich.edu (K.-L. Guan).

0898-6568/02/$ - see front matter © 2002 Elsevier Science Inc. All rights reserved.
doi:10.1016/S0898-6568(02)00139-0

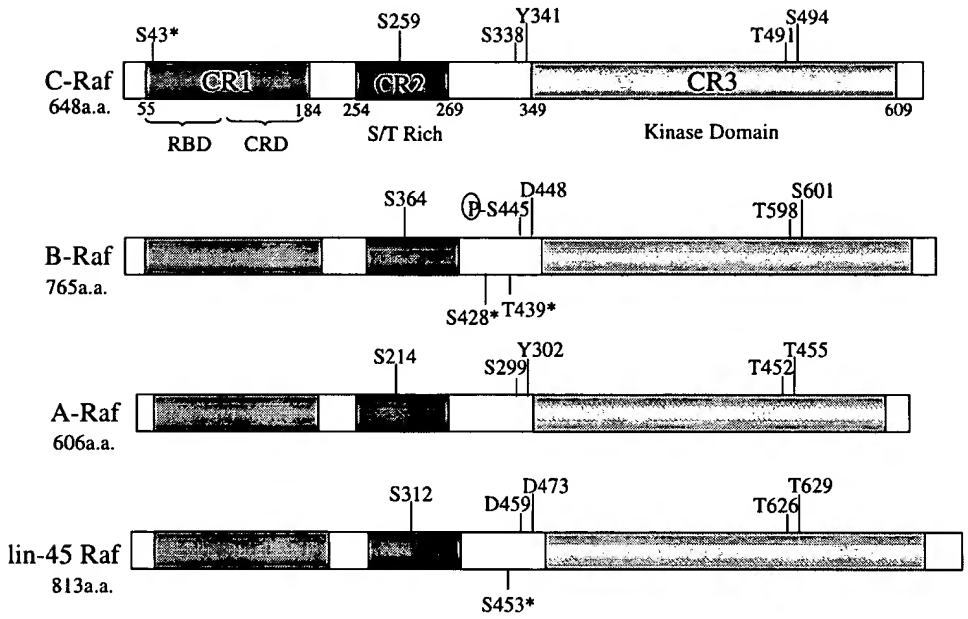

Fig. 1. The conserved structure of the Raf kinase family. The three conserved regions of Raf kinases are conserved across isoforms and species. CR1 has two Ras binding domains (RBD and CRD), CR2 is serine/threonine rich and CR3 is the catalytic kinase domain. Conserved regulatory phosphorylation sites are also shown. Significant regulatory sites that are not conserved but are acidic residues in other isoforms/species are noted. Other phosphorylation sites that are not conserved are marked with an asterisk (*). S43 and S259 in C-Raf, S364, S428 and T439 in B-Raf and S312 and S453 in lin-45 Raf are inhibitory phosphorylation sites. All other regulatory sites noted are activating phosphorylation sites.

forms. A-Raf is a much weaker activator of MEK compared to B- and C-Raf. Furthermore, A-Raf can only activate MEK1 where C-Raf is able to activate both MEK1 and MEK2 [24–26]. Although highly conserved at the primary sequences, Raf isoforms have similar and distinct biochemical and functional properties.

Raf isoforms also vary in their cell-specific expression and subcellular localization. For example, B-Raf (present as alternatively spliced isoforms) and C-Raf (expressed in many tissues) are expressed in the brain, while A-Raf is not [27]. A- and C-Raf are highly expressed in muscle, whereas B-Raf is detectable only at very low levels. In

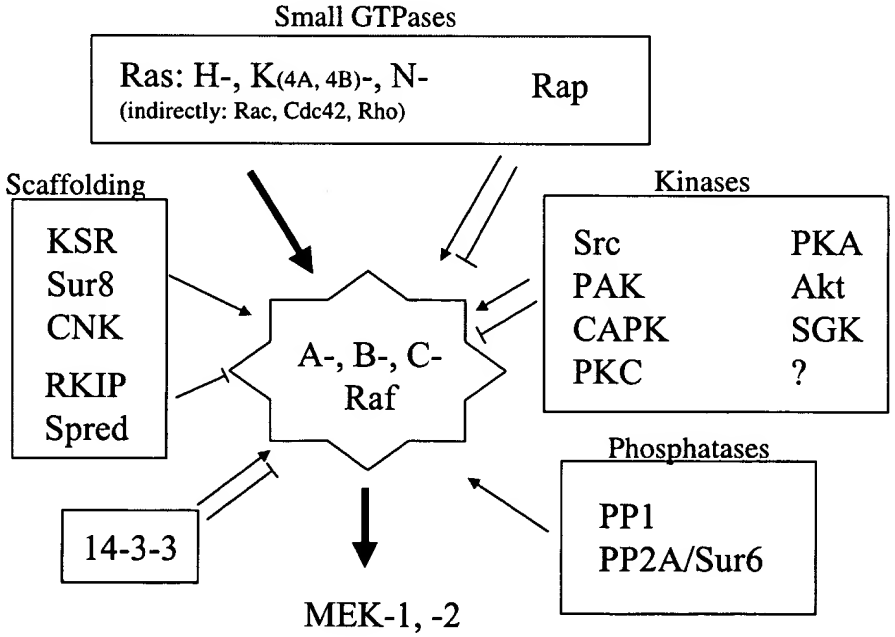

Fig. 2. Raf is a point of regulation in the MAP kinase pathway. Small GTPases, kinases, phosphatases, scaffolding proteins and 14-3-3 regulate the MAP kinase pathway through their effects on Raf kinase activity.

addition, Raf kinases are also localized to different subcellular compartments. Both A- and C-Raf have stable or transient localization to the mitochondria, which has implicated Raf in the regulation of apoptosis [28–31]. B- and C-Raf are both expressed in neurons; however, B-Raf is localized to neurite processes and C-Raf is perinuclear [27]. This localization may be due to isoform-specific lipid or protein-binding partners, which recruit Raf to distinct membrane rafts.

All Raf kinases are activated by the Ras small GTPase, regulatory phosphorylation events and scaffolding proteins (Fig. 2). The combination of all these events leads to the proper activation of Raf. Hence, the complexity of Raf activation lies in the fact that different Raf isoforms combine common and unique mechanisms to regulate Raf kinase activity.

2. Activation by small GTPases

Recruitment of Raf to the plasma membrane by the small GTPase Ras is the initial event in the Raf activation process. The effector domain of GTP-bound Ras binds to Raf through two domains in CR1, the Ras-binding domain (RBD) and the cysteine-rich domain (CRD) [3–5]. Binding to both sites is necessary for Raf activation. Although all Ras isoforms bind Raf with comparable affinity, different Ras isoforms activate Raf kinases to varying degrees. For example, K-Ras is a more potent activator of C-Raf than is H-Ras [32]. In addition, Raf activation by Ras is also affected by the subcellular localization of the various Ras isoforms through their prenylation signals, which function to target them to membranes of specific lipid content [33–35]. It has been recently shown that the prenylation signal on H- and N-Ras can localize these molecules to the ER and Golgi membrane, respectively [34]. Localization of Ras to these organelle membranes was shown to affect mitogen activated protein kinase (MAPK) signalling with kinetics different from signalling from the plasma membrane [34].

The small GTPase Rap has an identical effector domain to that of Ras, but whose activation results in different downstream functions [36]. Notably, Rap activates B-Raf but inhibits C-Raf activation. Rap binds the CRD of C-Raf with higher affinity than Ras [37] and excludes Ras from binding. Replacement of the CRD in C-Raf with the CRD from B-Raf results in the loss of Rap inhibition of C-Raf. Similarly, replacement of the CRD in B-Raf with the CRD from C-Raf results in the inhibition of B-Raf by Rap. The mechanism of Rap inhibition of C-Raf further suggests that small GTPase binding alone is insufficient for C-Raf activation.

The Rho family of small GTPases has been implicated in C-Raf activation. The Rho family, which consists of Rho, Rac and Cdc42, regulates cytoskeletal structures such as stress fibres, lamellipodia and filopodia, respectively. However, these GTPases do not directly bind Raf but signal via activation of downstream kinases. For example, Rho can signal through the activation of PKN1, PRK2 and ROCK1, where Rac and Cdc42 can signal through p21 activated kinase (PAK). Studies of C-Raf activation have led to the identification of a site phosphorylated by PAK (S338) that is critical to C-Raf activity [38–41]. Consistent with the role of PAK, the upstream activators Rac and Cdc42 cooperate with Ras to activate C-Raf [42]. One study showed that by using activated Ras mutants with an additional mutation that impairs C-Raf activation, but not Raf binding, activated Rac and Cdc42 were able to rescue C-Raf activation. However, activated Rac and Cdc42 alone could not induce C-Raf activity, and membrane localization of C-Raf was still required for activation. A dominant negative Rac mutant was able to abrogate Ras induction of C-Raf activity, which suggests that endogenous Rac is required for C-Raf activation. Similar observations were made with activated Rho; however, the kinase-activated downstream of Rho is unknown. Hence, it appears that the members of the Rho family of small GTPases are critical mediators which contribute indirectly to Ras-induced activation of Raf.

3. Phosphorylation

Phosphorylation is an important mechanism by which Raf activity is regulated. Most studies on Raf regulation have focused on C-Raf, but studies involving B-Raf, C-Raf and the *C. elegans* lin45-Raf have revealed that there is a conserved mechanism of regulation involving activation and inhibition by phosphorylation and dephosphorylation. Although the Raf kinases are structurally conserved between isoforms and species, there exist unique differences at key individual phosphorylation sites. Such differences explain how B- and C-Raf are both activated by Ras but can be regulated differently.

Ras activation induces phosphorylation of Raf at numerous residues; however, a number of residues are also phosphorylated in the basal, unstimulated state. These residues include S259 and S621 which were originally identified in C-Raf [7]. Phosphorylation of S259 and S621 creates two 14-3-3 binding sites which presumably help keep C-Raf in an auto-inhibited state. Phosphorylation at S621 may have more complex implications since mutation of this residue leads to complete inactivation of the kinase. Hence, a balance of phosphorylation and dephosphorylation is required to prime Raf properly in the basal state prior to stimulation by Ras or mitogens.

C-Raf has four activating phosphorylation sites that are Ras-inducible: S338, Y341, T491 and S494. S338 is the PAK phosphorylation site which lies just N-terminal to the kinase domain and is critical for Raf activation [15,38–43]. This site is conserved in the mammalian Rafs, as well as in *Drosophila* D-Raf. Interestingly, the homologous site in B-Raf, S445, is constitutively phosphorylated and may account for the high basal activity of B-Raf compared to

C-Raf [41]. *C. elegans* lin45-Raf has an aspartic acid residue substituted at this position which may mimic the effect of phosphorylation. Mutation of S338 in C-Raf results in a reduction of Raf activation induced by EGF, activated Ras, phorbol esters and the muscarinic G-protein coupled receptor pathway. Ras-induced phosphorylation of this site presumably occurs through Ras activation of PI3 kinase, whose lipid byproducts indirectly activate Rac exchange factors. Activated mutants of Rac and Cdc42 were also able to induce phosphorylation at S338, through activation of PAK.

Another C-Raf phosphorylation site is Y341 [24,41,44–47], which also lies just N-terminal to the kinase domain. Y341 is phosphorylated by Src and Janus activated kinase (JAK) [44,48,49]. In B-Raf, this tyrosine is replaced by an aspartic acid residue which can explain why B-Raf can be fully induced by Ras alone, but A- and C-Raf also require Src for full activation. A mutation in C-Raf that no longer binds Ras and therefore cannot be recruited to the membrane is also compromised in activation by Src [41,46]. Raf activation can be rescued if the mutant is artificially targeted to the plasma membrane where it can be activated by Src alone. However, Ras-mediated membrane recruitment and Src activation are not the only steps in C-Raf activation.

Two phosphorylation sites, T491 and S494, have been identified in the activation loop of the kinase domain of C-Raf [15]. These sites were first identified in B-Raf (T598 and S601), where mutation to alanine residues resulted in a loss of B-Raf activity induced by EGF and activated Ras, as well as by phorbol esters and muscarinic G-protein-coupled receptors [9]. Mutation of these sites in B-Raf to phosphomimetic residues resulted in constitutive activity independent of activated Ras. Recently, V599 in B-Raf was found to be frequently mutated to glutamic acid in human malignant melanomas and to a lower frequency in other cancers [50]. This tumour-associated mutation creates a negative charge next to the T598 phosphorylation site in the activation loop and is sufficient to activate B-Raf activity. The activation loop phosphorylation sites are also conserved across species. Acidic residue substitutions at homologous sites in lin45-Raf result in a multi-vulval phenotype indicative of an activated MAPK pathway [15]. The same acidic residue substitutions in C-Raf result in higher basal activity; however, Raf activity can be further enhanced by Ras through phosphorylation of S338 and Y341. T491 and S494 are phosphorylated in a Ras-dependent manner; however, the kinase that phosphorylates these residues has not been identified. Substitution of all four activating phosphorylation sites, S338, Y341, T491 and S494, to acidic residues results in full C-Raf kinase activity.

Phorbol esters, which activate protein kinase C (PKC), can activate Raf kinase activity. S499, which lies in the activation loop of C-Raf, was originally identified as a site phosphorylated by PKC [51]. However, several studies have found that mutation of S499 to alanine has no effect on PKC-stimulated Raf activity or Raf activity induced by activated Ras or EGF [15,52]. Hence, the role of phosphorylation of S499 as well as the mechanism of PKC-stimulated Raf activity still remains to be elucidated. A similar observation has occurred for S43 (located in the RBD) which was one of the first C-Raf phosphorylation sites identified [7]. S43 in C-Raf was thought to be directly phosphorylated by protein kinase A (PKA) and mediate PKA-induced inhibition [53]. However, it was shown that mutation of this site had no effect on PKA-induced C-Raf inhibition [54]. In B-Raf, this site is not conserved but B-Raf is still inhibited by forskolin-induced PKA [54,55]. Adding to the complexity of PKA-regulated Raf activity is the fact that PKA also stimulates Rap, which activates B-Raf [56]. The effect of PKA on C-Raf has been found to vary depending on cell type and serum conditions, which has led to confusion about the role of PKA and S43 in C-Raf regulation. This was clarified by the observation that during serum starvation, phosphorylation at S43 inhibits Ras binding to the RBD, and therefore inhibits C-Raf activation [57]. However, in the presence of mitogenic factors, C-Raf phosphorylated on S43 is able to bind Ras and thus this site plays no role in regulating C-Raf activity. The same study also showed that PKA can phosphorylate S43 as well as S259.

Inhibition by phosphorylation is a conserved mechanism of Raf regulation. In addition to S43, C-Raf contains an inhibitory phosphorylation site at S259. The current model suggests that phosphorylation of S259 along with phosphorylation of S621 creates an auto-inhibited conformation state that is maintained by a 14-3-3 dimer, thus bridging the N- and C-terminal domains together [3]. Akt was identified as the kinase that phosphorylates S259 and thus inhibits Raf activity [6,10,58]. Mutation of this site to alanine increases the basal activity of C-Raf. S259 is also conserved among the Raf isoforms as well as in *C. elegans* and *Drosophila* Raf kinases. However, in addition to the conserved S259 residue, B-Raf contains two additional Akt sites, S428 and T439. Unlike C-Raf, mutation of C-Raf S259 site in B-Raf (S364) to alanine results in a minor increase in basal activity [6,15]. Only when all three sites are mutated to alanine does the activity increase to a level comparable to Ras-stimulated activity. Similarly, single mutation of either of the two putative Akt sites to alanine in *C. elegans* lin-45 Raf has no phenotypic effect; however, mutation of both sites results in a mutli-vulval phenotype [15]. S364 in B-Raf has also been identified as a serum and glucocorticoid-inducible kinase (SGK) phosphorylation site [59]. SGK has some homology to Akt but, unlike Akt, preferentially targets S364 on B-Raf. Whether or not SGK regulation is unique to B-Raf or common to all Raf isoforms remains to be examined.

The serine/threonine phosphatases PP1 and PP2A have been shown to promote C-Raf activation [60–62]. The positive role of these phosphatases in the regulation of Raf activity was demonstrated initially in *C. elegans* [61]. A genetic screen identified Sur6, a subunit of PP2A, as an activator of the MAPK pathway. It was observed that the multi-vulval phenotype induced by activated Ras was

reduced by a mutation in Sur6, which suggests that Sur6 plays a positive role in signalling. These data also propose that the phosphatase acts downstream of Ras in the pathway. A study by Jaumot and Hancock [62] found that the critical target of PP1 and PP2A is S259. Mutation of S259 to alanine resulted in high basal activity. General and specific inhibitors of the PP1 and PP2A phosphatases abrogated Ras-stimulated C-Raf activity. Furthermore, inhibition of PP1 and PP2A resulted in a shift in C-Raf membrane microdomain localization and also in the increase of 14-3-3/C-Raf complexes at the plasma membrane. These complexes were not able to be activated even though they were localized to the plasma membrane. These data further support that membrane recruitment alone is insufficient for Raf activation and that PP1 and PP2A regulate microdomain localization of Raf which is important for Raf activity [62].

4. Scaffolding proteins

Genetic screens in *Drosophila* and *C. elegans* have revealed several novel genes that are important modulators of the Ras/MAPK pathway. One of these genes, called kinase suppressor of Ras (KSR), functions as a positive regulator and is proposed to act downstream of or in parallel to Ras [63–65]. KSR contains a C-terminal kinase domain with some homology to the Raf kinase family. However, the KSR kinase domain contains an arginine residue in place of a conserved catalytic lysine residue. Nevertheless, an initial report concluded that KSR does in fact possess kinase activity, is activated by ceramide and results in the direct phosphorylation of T269 of C-Raf to enhance activity [66]. Further observations showed that EGF can stimulate KSR activity (via an unknown mechanism) and that KSR mediates optimal Raf activation through phosphorylation of T269 [67,68]. However, several groups have been unable to detect any catalytic function for KSR and have proposed that KSR functions as a scaffolding protein based on its ability to interact with Raf, MEK, ERK, 14-3-3 and various heat shock proteins [69–72]. Expression of KSR in mammalian cells is able to redistribute MEK from the soluble fraction into a high molecular weight membrane-associated complex, suggestive of a function as a scaffolding protein [71]. A recent report suggests that KSR functions as a scaffold to facilitate the phosphorylation of MEK by Raf [73].

Observations made in *C. elegans* suggested that KSR kinase activity is not necessary for KSR function [71]. Predicted kinase-dead mutants of KSR were able to complement a KSR loss-of-function allele. However, recent analysis of the *C. elegans* genome has uncovered a second KSR gene, ksr-2 [74]. It was observed that only double mutants of ksr-1 and ksr-2 had strong defects in vulval development. This suggested that ksr-1 and ksr-2 are functionally redundant; however, both are essential for MAPK signalling in *C. elegans*. Ablation of the KSR gene in mouse resulted in a phenotypically normal animal in contrast to observations made in *C. elegans* [75]. However, the mouse did exhibit attenuated ERK activity and loss of the previously described high molecular weight scaffolding complex. In *Drosophila* S2 cells, reduction of KSR protein levels using RNAi impaired insulin-stimulated Raf activation [76]. Together, these studies suggest that although KSR may not be an essential component of MAPK signalling in mammals, it is required for enhanced MAPK signalling, hence supporting its role as a modulator.

Another product isolated from genetic screens is Sur8/Soc-2 [77,78]. Epistatic experiments have predicted Sur-8 to function downstream of Ras and upstream of Raf. Analysis of the Sur-8 sequence revealed multiple leucine-rich repeats which suggest its involvement in protein–protein interactions. Human Sur-8 is able to enhance Ras and EGF-stimulated Raf activity, but has no effect on Raf- or MEK-induced ERK activity [79]. Sur-8 is able to form a ternary complex with Ras and Raf, which suggests that its function is to facilitate activation by bringing these molecules into close proximity.

A third protein identified through genetic screens is *c*onnector e*n*hancer of *K*SR (CNK). Like KSR and Sur-8, epistatic studies in *Drosophila* place CNK downstream of Ras and parallel or upstream of Raf [80]. Using double-stranded RNA to knockdown expression, Anselmo et al. [76] showed that CNK and KSR in *Drosophila* S2 cells are required for Raf activation and that CNK specifically enhances the membrane recruitment of Raf. CNK has no apparent catalytic domain, but has multiple protein–protein interaction domains and localizes to cell–cell contact regions. CNK may be able to associate with other Ras-mediated, MAPK-independent pathways such as the RalGDS pathway [81]. The role of the mammalian CNK homologue, membrane-associated guanylate kinase-interacting protein (MAGUIN)-1, in Raf activation is still elusive [82].

The scaffolding proteins mentioned above enhance MAPK signalling. Raf kinase inhibitor protein (RKIP) is a scaffolding protein that inhibits the MAPK pathway [83]. Raf kinase inhibitor protein (RKIP) can bind Raf, MEK and ERK; however, the binding of Raf and MEK to RKIP is mutually exclusive [84]. The Raf and MEK binding sites on RKIP overlap and exclude each other from binding by steric interference. Suppression of endogenous RKIP expression was found to induce MEK/ERK activity. RKIP functions by inhibiting the formation of Raf and MEK in the same signalling module regardless of the activation state of Raf. Whether RKIP is specific to C-Raf or whether other isoforms have their own RKIP-like scaffolding inhibitor is unknown.

An even more potent inhibitor of the MAP kinase pathway is the Sprouty-related protein, Spred [85]. Sprouty was first identified in a genetic screen in *Drosophila* and was found to be a general inhibitor of receptor tyrosine kinases

[86,87]. Subsequently, Sprouty was shown to inhibit the formation of functional signalling complexes with the receptor and upstream of Ras [88]. Unlike Sprouty, Spred inhibits the MAP kinase pathway downstream of Ras and is able to associate with Raf. Although Spred binds Ras, Spred does not affect the activation of Ras or the recruitment of Raf to the membrane. However, it does inhibit Raf phosphorylation and activation by as yet undetermined mechanism [85]. The presence of enhancing and inhibitory scaffolding proteins adds another dimension to the diversity of Raf regulation.

5. Concluding remarks

The activation of Raf involves a complex series of events that include membrane recruitment, phosphorylation and dephosphorylation and the disruption and formation of various protein–protein interactions. Recent work has elucidated several new phosphorylation sites that are critical to the activation of Raf and also the signalling pathways that influence the phosphorylation state of Raf. In addition, genetic experiments in *Drosophila* and *C. elegans* have uncovered numerous Raf-binding proteins that scaffold the Raf complex and modulate its activation, which has added to the sheer complexity of the activation process. What has also become clear in recent years is that Raf isoforms share both common and unique mechanisms by which they are regulated. Since Raf isoforms have been shown to be expressed in different tissues and within various subcellular compartments, this suggests that it is not necessarily accurate to apply canonical mechanisms of Raf activation and function to each isoform. The complexity and diversity of Raf regulation continue to grow due to concerted efforts in the fields of genetics, biochemistry and cell biology. Future work in this area will undoubtedly take a more detailed analysis based on an isoform-, cell type- and stimulus-specific context of Raf signalling.

Acknowledgements

We would like to thank the members of the Guan Laboratory for helpful discussions. The authors are supported by the NIH National Research Service Award #5-T32-GM07544 (HC), the NIH, the Walthier Cancer Institute and a John D. and Katherine T. MacArthur Fellowship (KLG).

References

[1] Moelling K, Heimann B, Beimling P, Rapp UR, Sander T. Nature 1984;312(5994):558–61.
[2] Rapp UR, Goldsborough MD, Mark GE, Bonner TI, Groffen J, Reynolds Jr FH, et al. Proc Natl Acad Sci U S A 1983;80(14):4218–22.
[3] Morrison DK, Cutler RE. Curr Opin Cell Biol 1997;9(2):174–9.
[4] Nassar N, Horn G, Herrmann C, Scherer A, McCormick F, Wittinghofer A. Nature 1995;375(6532):554–60.
[5] Vojtek AB, Hollenberg SM, Cooper JA. Cell 1993;74(1):205–14.
[6] Guan KL, Figueroa C, Brtva TR, Zhu T, Taylor J, Barber TD, et al. J Biol Chem 2000;275(35):27354–9.
[7] Morrison DK, Heidecker G, Rapp UR, Copeland TD. J Biol Chem 1993;268(23):17309–16.
[8] Yao B, Zhang Y, Delikat S, Mathias S, Basu S, Kolesnick R. Nature 1995;378(6554):307–10.
[9] Zhang BH, Guan KL. EMBO J 2000;19(20):5429–39.
[10] Zimmermann S, Moelling K. Science 1999;286(5445):1741–4.
[11] Fukui M, Yamamoto T, Kawai S, Maruo K, Toyoshima K. Proc Natl Acad Sci U S A 1985;82(17):5954–8.
[12] Kasid U, Pfeifer A, Weichselbaum RR, Dritschilo A, Mark GE. Science 1987;237(4818):1039–41.
[13] Shimizu K, Nakatsu Y, Sekiguchi M, Hokamura K, Tanaka K, Terada M, et al. Proc Natl Acad Sci U S A 1985;82(17):5641–5.
[14] Stanton Jr VP, Cooper GM. Mol Cell Biol 1987;7(3):1171–9.
[15] Chong H, Lee J, Guan KL. EMBO J 2001;20(14):3716–27.
[16] Cutler Jr RE, Stephens RM, Saracino MR, Morrison DK. Proc Natl Acad Sci U S A 1998;95(16):9214–9.
[17] Heidecker G, Huleihel M, Cleveland JL, Kolch W, Beck TW, Lloyd P, et al. Mol Cell Biol 1990;10(6):2503–12.
[18] Huser M, Luckett J, Chiloeches A, Mercer K, Iwobi M, Giblett S, et al. EMBO J 2001;20(8):1940–51.
[19] Mercer K, Chiloeches A, Huser M, Kiernan M, Marais R, Pritchard C. Oncogene 2002;21(3):347–55.
[20] Mikula M, Schreiber M, Husak Z, Kucerova L, Ruth J, Wieser R, et al. EMBO J 2001;20(8):1952–62.
[21] Pritchard CA, Bolin L, Slattery R, Murray R, McMahon M. Curr Biol 1996;6(5):614–7.
[22] Wojnowski L, Zimmer AM, Beck TW, Hahn H, Bernal R, Rapp UR, et al. Nat Genet 1997;16(3):293–7.
[23] Wojnowski L, Stancato LF, Larner AC, Rapp UR, Zimmer A. Mech Dev 2000;91(1–2):97–104.
[24] Marais R, Light Y, Paterson HF, Mason CS, Marshall CJ. J Biol Chem 1997;272(7):4378–83.
[25] Pritchard CA, Samuels ML, Bosch E, McMahon M. Mol Cell Biol 1995;15(11):6430–42.
[26] Wu X, Noh SJ, Zhou G, Dixon JE, Guan KL. J Biol Chem 1996;271(6):3265–71.
[27] Morice C, Nothias F, Konig S, Vernier P, Baccarini M, Vincent JD, et al. Eur J Neurosci 1999;11(6):1995–2006.
[28] Majewski M, Nieborowska-Skorska M, Salomoni P, Slupianek A, Reiss K, Trotta R, et al. Cancer Res 1999;59(12):2815–9.
[29] Nantel A, Huber M, Thomas DY. J Biol Chem 1999;274(50):35719–24.
[30] Wang HG, Rapp UR, Reed JC. Cell 1996;87(4):629–38.
[31] Yuryev A, Ono M, Goff SA, Macaluso F, Wennogle LP. Mol Cell Biol 2000;20(13):4870–8.
[32] Yan J, Roy S, Apolloni A, Lane A, Hancock JF. J Biol Chem 1998;273(37):24052–6.
[33] Apolloni A, Prior IA, Lindsay M, Parton RG, Hancock JF. Mol Cell Biol 2000;20(7):2475–87.
[34] Chiu VK, Bivona T, Hach A, Sajous JB, Silletti J, Wiener H, et al. Nat Cell Biol 2002;4(5):343–50.
[35] Choy E, Chiu VK, Silletti J, Feoktistov M, Morimoto T, Michaelson D, et al. Cell 1999;98(1):69–80.
[36] Ohtsuka T, Shimizu K, Yamamori B, Kuroda S, Takai Y. J Biol Chem 1996;271(3):1258–61.
[37] Okada T, Hu CD, Jin TG, Kariya K, Yamawaki-Kataoka Y, Kataoka T. Mol Cell Biol 1999;19(9):6057–64.
[38] Chaudhary A, King WG, Mattaliano MD, Frost JA, Diaz B, Morrison DK, et al. Curr Biol 2000;10(9):551–4.

[39] Diaz B, Barnard D, Filson A, MacDonald S, King A, Marshall M. Mol Cell Biol 1997;17(8):4509–16.
[40] King AJ, Sun H, Diaz B, Barnard D, Miao W, Bagrodia S, et al. Nature 1998;396(6707):180–3.
[41] Mason CS, Springer CJ, Cooper RG, Superti-Furga G, Marshall CJ, Marais R. EMBO J 1999;18(8):2137–48.
[42] Li W, Chong H, Guan KL. J Biol Chem 2001;276(37):34728–37.
[43] Sun H, King AJ, Diaz HB, Marshall MS. Curr Biol 2000;10(5): 281–4.
[44] Fabian JR, Daar IO, Morrison DK. Mol Cell Biol 1993;13(11):7170–9.
[45] Jelinek T, Dent P, Sturgill TW, Weber MJ. Mol Cell Biol 1996;16(3): 1027–34.
[46] Marais R, Light Y, Paterson HF, Marshall CJ. EMBO J 1995;14(13): 3136–45.
[47] Stokoe D, McCormick F. EMBO J 1997;16(9):2384–96.
[48] Winston LA, Hunter T. J Biol Chem 1995;270(52):30837–40.
[49] Xia K, Mukhopadhyay NK, Inhorn RC, Barber DL, Rose PE, Lee RS, et al. Proc Natl Acad Sci U S A 1996;93(21):11681–6.
[50] Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, et al. Nature 2002;417(6892):949–54.
[51] Kolch W, Heidecker G, Kochs G, Hummel R, Vahidi H, Mischak H, et al. Nature 1993;364(6434):249–52.
[52] Barnard D, Diaz B, Clawson D, Marshall M. Oncogene 1998; 17(12):1539–47.
[53] Schramm K, Niehof M, Radziwill G, Rommel C, Moelling K. Biochem Biophys Res Commun 1994;201(2):740–7.
[54] Sidovar MF, Kozlowski P, Lee JW, Collins MA, He Y, Graves LM. J Biol Chem 2000;275(37):28688–94.
[55] Konig S, Guibert B, Morice C, Vernier P, Barnier JV. C R Acad Sci, Ser III 2001;324(8):673–81.
[56] Quilliam LA, Mueller H, Bohl BP, Prossnitz V, Sklar LA, Der CJ, et al. J Immunol 1991;147(5):1628–35.
[57] Dhillon AS, Pollock C, Steen H, Shaw PE, Mischak H, Kolch W. Mol Cell Biol 2002;22(10):3237–46.
[58] Rommel C, Clarke BA, Zimmermann S, Nunez L, Rossman R, Reid K, et al. Science 1999;286(5445):1738–41.
[59] Zhang BH, Tang ED, Zhu T, Greenberg ME, Vojtek AB, Guan KL. J Biol Chem 2001;276(34):31620–6.
[60] Abraham D, Podar K, Pacher M, Kubicek M, Welzel N, Hemmings BA, et al. J Biol Chem 2000;275(29):22300–4.
[61] Sieburth DS, Sundaram M, Howard RM, Han M. Genes Dev 1999; 13(19):2562–9.
[62] Jaumot M, Hancock JF. Oncogene 2001;20(30):3949–58.
[63] Kornfeld K, Hom DB, Horvitz HR. Cell 1995;83(6):903–13.
[64] Sundaram M, Han M. Cell 1995;83(6):889–901.
[65] Therrien M, Chang HC, Solomon NM, Karim FD, Wassarman DA, Rubin GM. Cell 1995;83(6):879–88.
[66] Zhang Y, Yao B, Delikat S, Bayoumy S, Lin XH, Basu S, et al. Cell 1997;89(1):63–72.
[67] Xing HR, Kolesnick R. J Biol Chem 2001;276(13):9733–41.
[68] Xing HR, Lozano J, Kolesnick R. J Biol Chem 2000;275(23): 17276–80.
[69] Cacace AM, Michaud NR, Therrien M, Mathes K, Copeland T, Rubin GM, et al. Mol Cell Biol 1999;19(1):229–40.
[70] Morrison DK. J Cell Sci 2001;114(Pt 9):1609–12.
[71] Stewart S, Sundaram M, Zhang Y, Lee J, Han M, Guan KL. Mol Cell Biol 1999;19(8):5523–34.
[72] Yu W, Fantl WJ, Harrowe G, Williams LT. Curr Biol 1998;8(1):56–64.
[73] Roy F, Laberge G, Douziech M, Ferland-McCollough D, Therrien M. Genes Dev 2002;16(4):427–38.
[74] Ohmachi M, Rocheleau CE, Church D, Lambie E, Schedl T, Sundaram MV. Curr Biol 2002;12(5):427–33.
[75] Nguyen A, Burack WR, Stock JL, Kortum R, Chaika OV, Afkarian M, et al. Mol Cell Biol 2002;22(9):3035–45.
[76] Anselmo AN, Bumeister R, Thomas JM, White MA. J Biol Chem 2002;277(8):5940–3.
[77] Selfors LM, Schutzman JL, Borland CZ, Stern MJ. Proc Natl Acad Sci U S A 1998;95(12):6903–8.
[78] Sieburth DS, Sun Q, Han M. Cell 1998;94(1):119–30.
[79] Li W, Han M, Guan KL. Genes Dev 2000;14(8):895–900.
[80] Therrien M, Wong AM, Rubin GM. Cell 1998;95(3):343–53.
[81] Therrien M, Wong AM, Kwan E, Rubin GM. Proc Natl Acad Sci U S A 1999;96(23):13259–63.
[82] Yao I, Ohtsuka T, Kawabe H, Matsuura Y, Takai Y, Hata Y. Biochem Biophys Res Commun 2000;270(2):538–42.
[83] Yeung K, Seitz T, Li S, Janosch P, McFerran B, Kaiser C, et al. Nature 1999;401(6749):173–7.
[84] Yeung K, Janosch P, McFerran B, Rose DW, Mischak H, Sedivy JM, et al. Mol Cell Biol 2000;20(9):3079–85.
[85] Wakioka T, Sasaki A, Kato R, Shouda T, Matsumoto A, Miyoshi K, et al. Nature 2001;412(6847):647–51.
[86] Hacohen N, Kramer S, Sutherland D, Hiromi Y, Krasnow MA. Cell 1998;92(2):253–63.
[87] Reich A, Sapir A, Shilo B. Development 1999;126(18):4139–47.
[88] Casci T, Vinos J, Freeman M. Cell 1999;96(5):655–65.

VOLUME 23 · NUMBER 27 · SEPTEMBER 20 2005

JOURNAL OF CLINICAL ONCOLOGY BIOLOGY OF NEOPLASIA

Raf: A Strategic Target for Therapeutic Development Against Cancer

Muralidhar Beeram, Amita Patnaik, and Eric K. Rowinsky

From The University of Texas Health Science Center at San Antonio and Institute for Drug Development, Cancer Therapy and Research Center, San Antonio, TX.

Submitted July 30, 2004; accepted July 14, 2005.

The authors have participated as investigators in several clinical studies of sorafenib funded by Bayer Corporation Pharmaceutical Division, New Haven, CT.

Authors' disclosures of potential conflicts of interest are found at the end of this article.

Address reprint requests to Eric K. Rowinsky, MD, 11710 Spotted Eagle, San Antonio, TX, 78248; e-mail: erowinsk@oncodrugs.com.

0732-183X/05/2327-6771/$20.00

DOI: 10.1200/JCO.2005.08.036

ABSTRACT

The mitogen-activated protein kinase (MAPK) signaling pathway plays a critical role in transmitting proliferative signals generated by cell surface receptors and cytoplasmic signaling elements to the nucleus. Several important signaling elements of the MAPK pathway, particularly Ras and Raf, are encoded by oncogenes, and as such, their structures and functions can be modified, rendering them constitutively active. Because the MAPK pathway is dysregulated in a notable proportion of human malignancies, many of its aberrant and critical components represent strategic targets for therapeutic development against cancer. Raf, which is an essential serine/threonine kinase constituent of the MAPK pathway and a downstream effector of the central signal transduction mediator Ras, is activated in a wide range of human malignancies by aberrant signaling upstream of the protein (eg, growth factor receptors and mutant Ras) and activating mutations of the protein itself, both of which confer a proliferative advantage. Three isoforms of Raf have been identified, and therapeutics targeting Raf, including small-molecule inhibitors and antisense oligodeoxyribonucleotides (ASON), are undergoing clinical evaluation. The outcomes of these investigations may have far-reaching implications in the management of many types of human cancer. This review outlines the structure and diverse functions of Raf, the rationale for targeting Raf as a therapeutic strategy against cancer, and the present status of various therapeutic approaches including ASONs and small molecules, particularly sorafenib (BAY 43-9006).

J Clin Oncol 23:6771-6790. © 2005 by American Society of Clinical Oncology

INTRODUCTION

The *ras* family of oncogenes and encoded proteins has been evaluated as a putative target for anticancer therapeutic development. These efforts have resulted in new insights into Ras-mediated cell signaling as it relates to human cancer. Ras plays a central role in an intricate array of signal transduction pathways, characterized by cross talk, feedback loops, and multicomponent signaling complexes.[1-3] One strategy to overcome the challenges inherent in developing therapeutics against signaling elements situated in redundant pathways is to target elements downstream of convergence points of critical signaling modules. This reasoning has led, in part, to interest in Raf kinase, which is one of several downstream effectors of Ras, as a target for therapeutic development against cancer.

The Raf serine/threonine kinases are the principal effectors of Ras in the mitogen-activated protein kinase (MAPK) pathway (Fig 1). Raf activation occurs immediately downstream of membrane and cytoplasmic receptors that relay mitogenic signals.[4] Although principally activated by Ras, Raf may also be activated by Ras-independent elements and, in turn, propagates signals through diverse effectors that mediate proliferation, angiogenesis, metastases, and survival.[5] Raf may be activated by signaling upstream or constitutively. Constitutive activation of Raf and Ras are indistinguishable in their potential to induce malignant transformation.[6-8] Activating *raf* mutations have been identified in melanoma, thyroid, colon, and other cancers (Table 1).[9-43] Furthermore, the disappointing clinical results of farnesyltransferase (FTase) inhibitors (FTIs) that were developed based on a flawed premise that they would effectively target malignancies with a high incidence of Ras mutation has led to scrutiny of signaling elements downstream of Ras, such

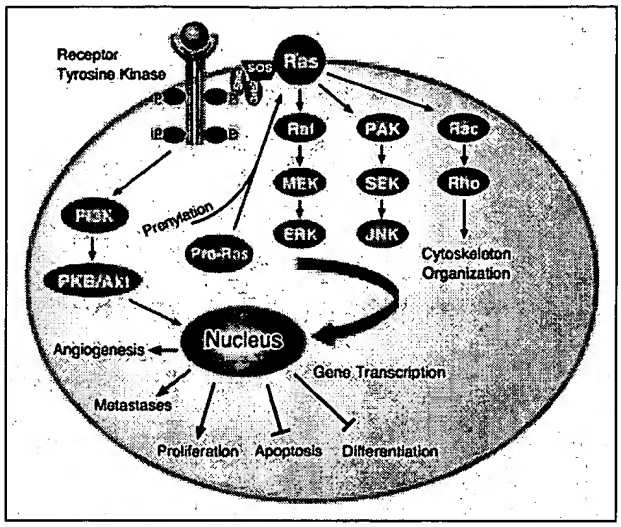

Fig 1. Ras-mediated signal transduction pathways. Abbreviations: PKB/Akt, protein kinase B; ERK, extracellular signal–regulated kinase; Grb2, growth factor receptor binding protein; JNK, c-JUN amino-terminal kinase; MEK, mitogen-activated protein kinase kinase; P, phosphate; PAK, p21-activated kinase kinase or JNK kinase; PI3K, phosphatidylinositol 3-kinase; Shc, Src homology domain-cytosol; SEK, stress-activated protein kinase; and SOS, son-of-sevenless exchange factor.

as Raf, as therapeutic targets.[44] This review highlights relevant information about the biology of Raf and novel strategies directed at exploiting this knowledge to more effectively treat malignant diseases.

SIGNALING THROUGH THE MAPK PATHWAY

The molecular mechanisms and signaling pathways that regulate cell proliferation and survival are receiving considerable attention as potential targets for anticancer strategies.[45,46] Recently, there has been a notable increase in efforts directed at targeting the MAPK pathway, which integrates a wide array of proliferative signals initiated by receptor tyrosine kinases (RTKs) and G protein–coupled receptors.[47,48] The network of signals emanating from the MAPK pathway are transmitted by proteins that serve as chemical switches, cycling between phosphorylated (activated) and dephosphorylated (inactivated) states.[49] These on and off switches are regulated by kinases and phosphatases, respectively. Activated signaling elements, in turn, phosphorylate amino acid residues on downstream signaling proteins in a cascade-like and expansive manner.[50] Not only does the centrality of the MAPK pathway render its components important targets for therapeutic development, but many genes that encode for its critical signaling elements undergo mutations, constitutively activating downstream signaling elements and conferring the potential for transformation and autonomous growth.[50-52] In addition to efforts directed at Ras, therapeutic strategies directed at the MAPK pathway are targeting the cascade of downstream effector proteins including Raf, MAPK kinase (MAPKK; also called MEK or extracellular signal–regulated kinase [ERK] kinase), and ERK. The Raf/MEK/ERK module (Fig 1) of the MAPK pathway, which is immediately downstream of Ras, may be less redundant and innately resistant to therapeutic manipulations compared with Ras (as discussed in the next section).

The Unfilled Promise of Targeting Ras

The Raf/MEK/ERK module of the MAPK pathway has been the focus of considerable attention because therapeutic efforts directed at Ras, which is situated at the apex of the MAPK pathway, have been disappointing.[44] Ras belongs to a superfamily of guanine nucleotide triphosphatases (GTPases) that transmit proliferative and survival signals to the MAPK, phosphatidylinositol 3-kinase (PI3K), and other pathways (Figs 1 and 2). Three *ras* proto-oncogenes encode four 21-kd proteins, called $p21^{ras}$ or Ras (H-Ras, N-Ras, K-Ras4A, and K-Ras4B, resulting from two alternatively spliced *K-Ras* gene products), that are localized to the inner surface of the cell membrane.[44] Of the three *ras* genes, *K-ras* mutations are most commonly found in solid malignancies, whereas *N-ras* mutations are encountered less often, and *H-ras* mutations are rarely encountered.[53,54] Ras isoforms impart distinct biologic effects as a result of the potential of these proteins to differentially activitate critical effectors.[55]

After synthesis as inactive cytosolic propeptides, Ras undergoes a series of post-translational modifications at its carboxyl terminus that increase its hydrophobicity.[56,57] These modifications render Ras functional and capable of localizing to the lipid-rich inner surface of the cell membrane. The first and most critical modification, farnesylation, which is principally catalyzed by protein FTase, adds a 15-carbon hydrobobic farnesyl isoprenyl tail to the carboxyl terminus of Ras. It is in the cell membrane where Ras cycles between inactive guanosine diphosphate–bound and active guanosine triphosphate (GTP)–bound states, thereby activating a series of effector kinases that phosphorylate a cascade of signaling proteins.[58] Ras mutants exhibit slightly less intrinsic GTPase activity than wild-type Ras; however, the principal consequence of the mutated proteins is a marked decrease in interactions between Ras and its GTPase activator protein.[59] Instead of reverting to its inactive guanosine diphosphate–bound state, the modified conformation of mutant Ras favors its active GTP-bound state, which has a higher propensity to activate downstream effectors even in the absence of growth factor stimulation, conferring a proliferative advantage to tumors.

The considerable attention paid to targeting Ras as a therapeutic strategy is based on the high incidence of activating *ras* mutations in human malignancies, including

Table 1. *raf* Alterations and Mutations and Human Cancers

Raf Isoform	Predominant Genetic Alterations	Type of Malignancy	Reference	Frequency of Raf Mutation (%)*	Frequency of Ras Mutation (%)
B-Raf	Point (missense) mutations	Malignant melanoma	Davies et al[9] and Pollock and Meltzer[17]	55-68	16
		Anaplastic thyroid carcinoma	Nikiforova et al[15]	83	—†
		Papillary thyroid carcinoma	Kimura et al[16] and Cohen et al[18]	35.8-69	25
		Cholangiocarcinoma	Tannapfel et al[21]	0-21	56
		Colorectal carcinoma	Rajagopalan et al[14]	4-16	36
		Esophageal carcinoma, Barrett's	Sommerer et al[12]	15	—†
		Acute myeloid leukemia	Lee et al[22]	4	23
		Head and neck carcinoma, squamous	Cohen et al[19] and Weber et al[23]	3-4.8	23
		Lung carcinoma, non–small-cell	Brose et al[24]	2-3	22
		Gastric carcinoma	Lee et al[25]	2	—†
		Ovarian carcinoma, low-grade/high-grade	Singer et al[20]	63/0	23
		Mucinous ovarian carcinoma	Gemignani et al[26]	0	50
		Non-Hodgkins lymphoma	Lee et al[27]	2	—†
C-Raf	Gene rearrangements; point mutations; truncated amino-terminal regulatory domain	Renal cell carcinoma	Oka et al[28]	55	10
		Medullary thyroid carcinoma	Carson et al[29]	—†	—†
		Breast carcinoma	Callans et al[13] and McFarlin and Gould[30]	—†	2
		Lung carcinoma, non–small-cell	Kerkhoff et al[31]	—†	22
		Lung carcinoma, small-cell	Graziano et al[32]	> 90	< 2
		Head and neck carcinoma, squamous	Patel et al[33] and Riva et al[34]	—†	23
		Soft tissue and bone sarcomas	Ikeda et al[35] and Mitsunobu et al[36]	—†	—†
		CNS: glioma, glioblastoma, ependymoma	LaRocca et al,[37] Fukui et al,[38] and Korshunov et al[39]	—†	—†
		Hepatocellular carcinoma	Ting et al,[40] Jenke et al,[41] and Beer et al[42]	—†	31
		Pancreatic carcinoma	Berger et al[43]	—†	78
		Non-Hodgkins lymphomas, T-cell	Storm and Rapp[10]	—†	—†

*Frequency values based on a review of the literature, largely encompassing small and moderately sized studies that generally surveyed tumor biopsy samples in a retrospective manner.
†The precise frequency of the specific genetic alteration is not known. References discuss phenomena but relate to small numbers of patients.

approximately 22% of non–small-cell lung, 50% of colorectal, and 90% of pancreatic cancers.[53,60,61] Of the strategies directed at Ras, targeting FTase has received the most attention, but the FTIs are not Ras specific, and a bonafide Ras-specific therapeutic agent has not yet been evaluated in clinical trials.[62,63] Fortunately, because *K-ras* mutations constitute most *ras* mutations in the aforementioned malignancies, in which the therapeutic expectations of FTIs were among the highest, the failure of this strategy should not be surprising because geranylgeranyl transferase I can alternatively prenylate *K-ras*, rendering it functional even when FTase is completely inhibited.[64,65] Although the FTIs have shown notable antitumor activity in patients with advanced breast cancer and some hematologic malignancies, the low *ras* mutation rates in these cancers suggest that farnesylation of other critical proteins is being inhibited.[66]

DOWNSTREAM OF RAS: RAF AND OTHER RAS EFFECTORS

Localization of GTP-bound Ras to the inner surface of the cell membrane activates several downstream effectors, most notably the serine/threonine kinase Raf, which is the first signaling element in the MAPK pathway.[2,67,68] As shown in Figure 1, other downstream effectors of Ras include the PI3K cell survival pathway, the small GTP-binding proteins Rac and Rho, and the stress-activated protein kinase pathway (also referred to as the c-jun N-terminal kinase [JNK] pathway).[69-71] In addition, in response to cellular stress and cytokine stimulation mediated through Ras, the dual-specificity $p38^{MAPK}$ kinases (MKK3 and MKK6) and the JNK kinases (MKK4 and MKK7) phosphorylate $p38^{MAPK}$ and JNK, respectively.[72-76]

GTP-bound Ras interacts directly with Raf and mobilizes the inactive protein from the cytoplasm (Figs 1 and 2).

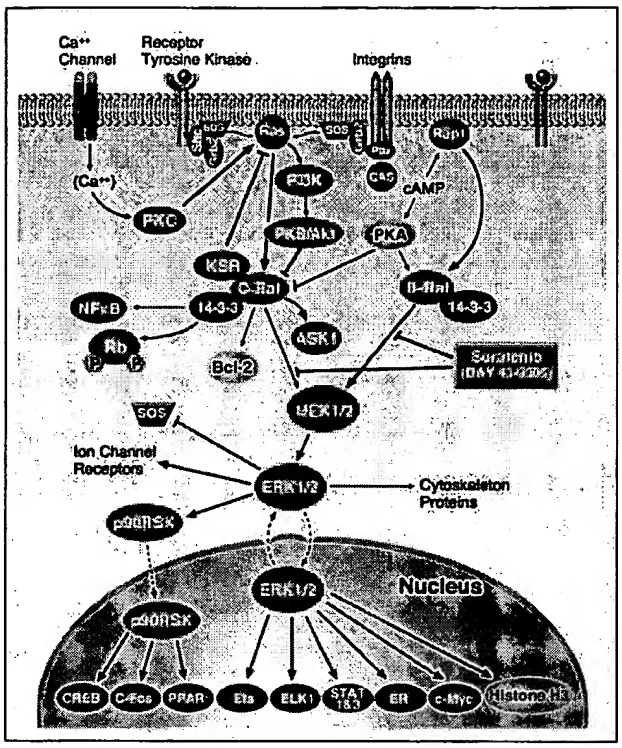

Fig 2. Raf is stimulated by diverse mitogenic stimuli and, in turn, activates multiple effectors. Abbreviations: ASK1, apoptosis signal-regulated kinase; CREB, cyclic adenosine monophosphate response element B; c-Fos, c-Myc, Ets, and ELK1, transcription factors; NF-κB, nuclear factor-kappa B; p90RSK, 90-kd ribosomal S6 kinase; PPARγ, peroxisome proliferator activated receptor gamma; Rb, retinoblastoma protein; Shc, Src homology domain-cytosol; STAT, signal transducer and activator of transcription.

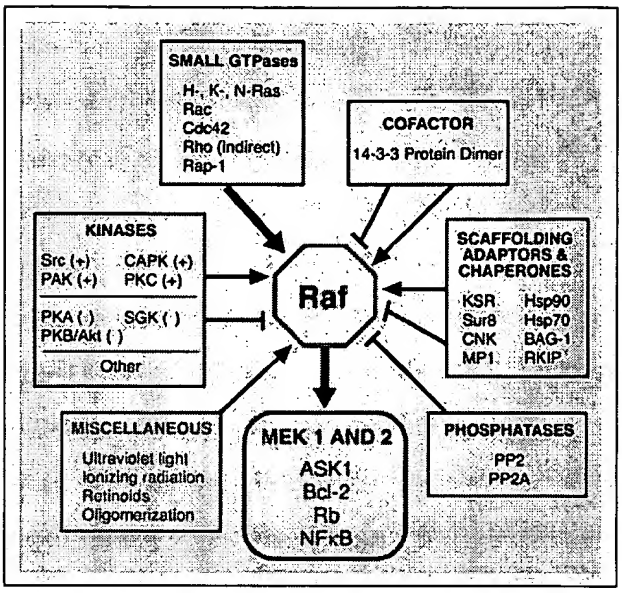

Fig 3. The activating and inhibitory stimuli converging on Raf and its principal downstream effectors. Abbreviations: ASK1, apoptosis signal-regulated kinase; BAG1, Bcl2- associated athanogene; CAPK, ceramide-activated protein kinase; cdc42, cyclin-dependent kinase; Hsp, heat shock proteins; KSR, kinase suppressor of Ras; MP1, MEK partner-1; NF-κB, nuclear factor-kappa B; PAK, p21-activated kinase; PKC, protein kinase C; PKB/Akt, protein kinase B; PP1 and PP2A, protein phosphatases; Rap1, repressor activator protein 1; Rb, retinoblastoma protein; RKIP, Raf kinase–inhibitor protein; SGK, steroid glucocorticoid kinase; Src, soluble nonreceptor tyrosine kinase; (+), activator; (−), inhibitor.

Once the Ras-Raf complex is translocated to the cell membrane, Ras activates the serine/threonine kinase function of Raf through an association between its Ras-binding domain (RBD) in the amino-terminal regulatory region and Ras-GTP. This is followed by a series of Ras-dependent phosphorylation events and conformational changes, which will be described later in this review.[77-84] The regulatory mechanisms of various Raf isoforms differ in that A-Raf and C-Raf require additional phosphorylation reactions for activity, whereas B-Raf has a much higher level of basal kinase activity.[85]

Raf is also activated by Ras-independent activators, including the soluble non-RTK Src and Janus kinase 1, which are involved in cytokine signaling.[86] Other Ras-independent activators of Raf include interferon beta, protein kinase C (PKC) alpha, antiapoptotic proteins (eg, Bcl-2), scaffolding proteins (eg, ceramide-activated protein kinase), ultraviolet light, ionizing radiation, retinoids, erythropoietin, and dimerization between Raf isoforms[86-94] (Fig 3). In addition, several *Raf* mutations confer constitutive activity to Raf irrespective of signaling activity upstream.[9,11,12] The multifactoral mechanisms of Raf activation imply that therapeutic strategies that depend on the abrogation of any single element of these pathways may not result in sufficiently robust tumor growth–inhibitory activity. Furthermore, the kinase activity of Raf is inhibited by its interactions with cholesterol-rich lipid rafts in the cell membrane and phosphorylation by protein kinases A (PKA) and B (PKB/Akt), as shown in Figure 2.[95-98] In essence, the activation status of Raf depends on the integration of both activating and inhibitory stimuli, the net result of which determines the downstream messages.

THE RAF FAMILY OF GENES AND PROTEINS

The *raf* family of genes was first identified as oncogenes in retroviruses that are the causative vectors of tumors in mice and chicken.[99,100] The first *raf* gene to be identified, *v-raf*, the transforming gene of the mouse sarcoma virus 3611, induces fibrosarcomas and erythroleukemia in newborn mice, and *C-raf* (also called *raf-1*) is its proto-oncogene homolog.[101,102] *A-raf* was found next by screening a mouse spleen cDNA library at low stringency with a *v-raf* probe. Next, *v-Rmil* was identified as the transforming gene in the avian retrovirus Mill Hill No. 2 (MH2) from a spontaneous ovarian tumor and found to be homologous to *v-raf*.[99] *C-Rmil* corresponded to a third mammalian *raf* gene, *B-raf*, which was also shown to be an oncogene.[103,104] However, initial attempts to identify activated versions of *raf* in human cancers failed to demonstrate unique DNA rearrangements in

any specific tumor type, which, in retrospect, can be attributed to the lack of requisite sensitivity of early assays to detect single point mutations. Furthermore, most early efforts were directed at *C-raf*, rather than at the more oncogenic *B-raf*.

The mammalian *raf* family consists of the following three genes: *A-raf*, *B-raf*, and *C-raf*, which are located on chromosomes Xp11, 7q32, and 3p25, respectively. The *raf* proto-oncogenes encode three 68- to 74-kd cytosolic proteins, termed A-Raf, B-Raf, and C-Raf (Raf-1), which share highly conserved amino-terminal regulatory regions and catalytic domains at the carboxyl terminus (Fig 4).[10] As serine/threonine kinases, Raf proteins phosphorylate serine and threonine residues on essential modulatory proteins downstream of Ras. Each Raf species has a distinct expression profile in tissues, which suggests that individual Raf isoforms perform clearly defined functions.[4] C-Raf is ubiquitously expressed in most tissues. Both A- and B-Raf have more restricted expression profiles than C-Raf, with A-Raf overexpressed in urogenital tissues (eg, kidney, ovary, prostate, and epididymis) and B-Raf overexpressed in neural, testicular, splenic, and hematopoietic tissues.[105] Unlike *A-raf* and *C-raf*, *B-raf* undergoes differential splicing in exons 8b and 10a, and its spliced variants are translated into 10 B-Raf isoforms.[106,107] Both A-Raf and C-Raf undergo localization to the mitochondria, which supports the notion that Raf regulates apoptosis, but the specific proportions of Raf isoforms that are localized to the mitochondria are not known.[108-112] This localization may be a result of isoform-specific lipid- or protein-binding partners, which recruit Raf to distinct membrane rafts.

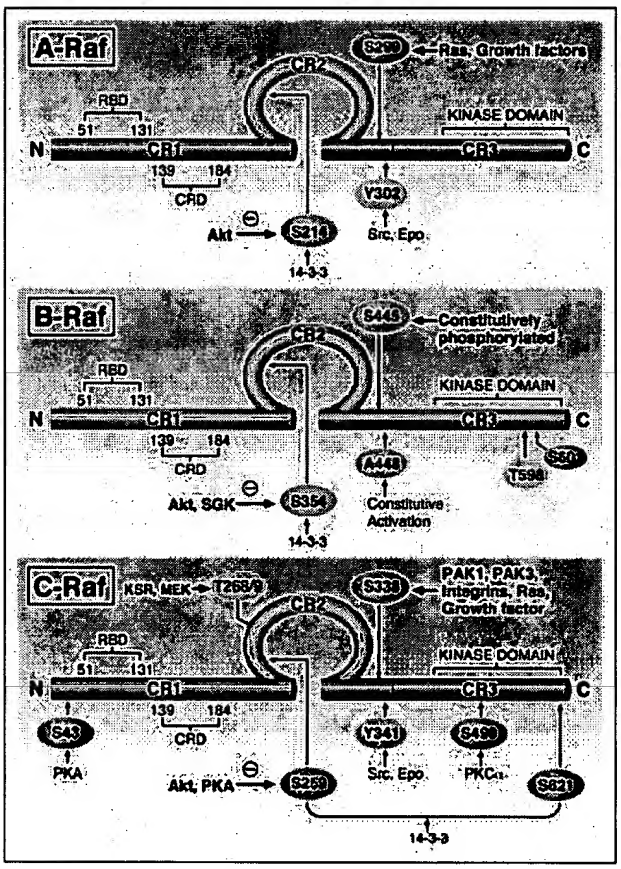

Fig 4. Schema of the domain structure of A-Raf, B-Raf, and C-Raf. The amino acid phosphorylation sites (S, serine; T, threonine; Y, tyrosine) and phosphorylating stimuli regulating the Raf kinases are shown. Abbreviations: C, N, carboxyl and amino terminus; RBD, Ras-binding domain; CRD, cysteine-rich domain; Epo, erythropoietin; KSR, kinase suppressor of Ras; MEK, mitogen-activated protein kinase kinase; PAK, p21-activated kinase; PKA, protein kinase A; PKCα, protein kinase C alpha; SGK, steroid glucocorticoid kinase; Src, soluble nonreceptor tyrosine kinase. (Reproduced from Dowsett et al: Short-term changes in Ki-67 during neoadjuvant treatment of primary breast cancer with anastrozole or tamoxifen alone or combined correlate with recurrence-free survival. Clin Cancer Res 11:951S-958S, 2005)

From a functional standpoint, although all Raf proteins are serine/threonine kinases and capable of activating the MAPK cascade, they have distinct downstream phosphorylation targets and play unique roles in signaling.[113] Their distinct roles are supported by *Raf* knockout studies, in which mice lacking each of the three Raf proteins have disparate phenotypes.[113,114] *B-raf* knockouts die in utero by day 12, usually as a result of massive internal hemorrhage, whereas *A-raf* and *C-raf* knockouts die postpartum with extensive intestinal distension (*A-raf* knockout) or failure of lung maturation (*C-raf* knockout). Additional support for the diverse functionality of Raf family members is provided by the disparate responses of B-Raf and C-Raf to identical stimuli, as well as the distinct messages that each isoform relays downstream to Rap1, which is a small GTPase that functions as both an activator and repressor of Raf.[115] For example, Rap1-mediated stimulation of B-Raf by cyclic adenosine monophosphate (cAMP) phosphorylates ERK, whereas stimulation of C-Raf inhibits ERK phosphorylation.[115]

The Structure of Raf

The structure of Raf consists of the following: (1) an amino terminus that contains the regulatory domain; (2) an activation loop; and (3) a carboxyl terminus that contains the kinase domain[116-118] (Fig 4). All Raf kinases are composed of three conserved regions, CR1 (adjacent to the amino terminus), CR2, and CR3 (adjacent to the carboxyl terminus). The regulation of Raf kinase activity is a complex process involving phosphorylation of the regulatory and catalytic domains of the protein and both inter- and intramolecular interactions. The initial process of Raf activation involves the interaction of active GTP-bound Ras with the RBD of Raf and the adjacent zinc-binding cysteine-rich domain (CRD) of CR1, facilitating recruitment of Raf to the cell membrane for activation.[78] The role of CR2, which is rich in serine and threonine residues, is less well defined; however, the phosphorylation of moieities within CR2 and various protein-protein interactions involving CR2 also affect Raf localization and activation.[89,119-121] Deletions of the amino-terminal regulatory domains CR1 and CR2, similar to *v*-Raf, are found in

several types of human cancers with activating *Raf* mutations, suggesting that these domains negatively regulate Raf function. CR3, the catalytic domain of Raf, is also subject to regulation by phosphorylation.

Regulation of Raf Kinase Activity

General. The overlapping functional aspects of the three Raf isoforms have been elucidated by studies involving Raf knockout mice. In C-Raf knockouts, B-Raf can compensate for the loss of C-Raf in activating MEK in the MAPK pathway, but C-Raf knockouts are much more susceptible to apoptotic stimuli, despite the presence of A-Raf and B-Raf.[122] With regard to differences in signaling between the Raf isoforms, A-Raf is a weaker activator of MEK than B-Raf or C-Raf. Furthermore, A-Raf can activate MEK1 only, whereas C-Raf activates both MEK1 and MEK2.[123-125] As shown in Figure 3, Raf kinases are activated by Ras, other small GTPase regulatory proteins, and scaffolding proteins, and the magnitude and quality of downstream signaling are dependent on the integration of activating events and protein interactions.

C-Raf exists in the cytoplasm as a 300- to 500-kd protein complex. The complex consists of C-Raf, heat shock protein 90 (Hsp90), and the dimeric protein cofactor 14-3-3. 14-3-3 binds to two specific phosphoserine residues of C-Raf, which masks its kinase domain and inactivates the protein. The binding of Ras to C-Raf displaces the 14-3-3 dimer, rendering C-Raf accessible to dephosphorylation by protein phosphatase 2A.[126] This action enables subsequent activation of C-Raf by mitogenic stimuli. The stability and function of C-Raf are also regulated by the phosphorylation status of C-Raf itself, the binding of C-Raf to Ras, and interactions between C-Raf and cellular lipids.

Activation by Ras and other small GTPases. The initial event in the activation of Raf is its recruitment to the inner surface of the cell membrane by the small GTPase Ras. The effector domain of GTP-bound Ras binds to C-Raf through the RBD and CRD in the CR1. Although binding to both sites is required for Raf activation, the most critical interaction is between Ras-GTP and the RBD.[127] All Ras isoforms are capable of interacting with Raf, but K-Ras is the most potent activator, whereas N-Ras is much more efficient than H-Ras.[128] The interaction between Ras and C-Raf alone is insufficient to activate C-Raf, but it serves to translocate C-Raf to the cell membrane where it can be activated.

The activation of B-Raf by Ras has been less well studied; however, the interacting amino acids in the Ras-Raf interface are identical for B-Raf and C-Raf.[129,130] The association of Ras with B-Raf also translocates B-Raf to the cell membrane where it is activated.[124] Interestingly, a membrane-free complex of B-Raf and 14-3-3 can be activated in vitro by recombinant Ras. This is in stark contrast to A-Raf and C-Raf, which must undergo a series of phosphorylation reactions on serine and tyrosine residues in the cell membrane and cannot be activated by Ras alone.[124,130] Of the Raf isoforms, B-Raf is activated first, and on stimulation by Ras, it heterodimerizes with C-Raf, the significance of which is not known.[94]

Both B-Raf and C-Raf can bind to other small GTPases, most notably Rap1.[115,131,132] The effector domains of Rap1 and Ras are nearly identical, but activation of these proteins produces vastly different downstream effects. Furthermore, Rap1 mediates distinct effects after binding to various Raf isoforms. The B-Raf–Rap1 complex activates B-Raf, whereas the C-Raf–Rap1 interaction does not activate C-Raf and, in fact, may be inhibitory.[115,132,133] This occurs because Rap1 binds to the CRD of C-Raf with higher affinity than Ras and excludes Ras from binding.

The Rho family of small GTPases, consisting of Rho, Rac, and cyclin-dependent kinase (Cdc) 42, regulate cytoskeletal organization during the cell cycle and also mediate Ras-induced activation of Raf, especially C-Raf.[134-136] These GTPases do not directly bind to Raf but, instead, signal by activating downstream kinases. Rho signals by activating the serine/threonine protein kinases N1 and N2 and Rho-associated kinase 1, whereas Rac and Cdc42 signal through p21-activated kinase (PAK).[134-136]

Phosphorylation. Raf is principally activated by phosphorylation of specific amino acid residues as shown for each isoform in Figure 4. From an evolutionary standpoint, the Raf activation sites are highly conserved from yeast to humans. Several amino acids in Raf, particularly serine (S) 259 and S621, which bind 14-3-3 and maintain C-Raf in a closed auto-inhibited conformation, are phosphorylated in the basal state.[137] On stimulation, Ras-GTP displaces 14-3-3 from S259, and C-Raf is translocated to the cell membrane, where it can be dephosphorylated at S259 by protein phosphatase 2A or other phosphatases.[126] S259 also represents the site of inhibitory phosphorylation by PKB/Akt, PKA, and serum glucocorticoid-inducible kinase.[121,138,139] Phosphorylation at S621 seems to have greater significance because mutations at this site inactivate Raf's kinase activity. Hence, a balance of phosphorylation and dephosphorylation is required to prime Raf in the basal state before stimulation by Ras or mitogens.[137]

Raf is also phosphorylated at other serine and threonine residues, the most important of which are S338 and tyrosine (Y) 341, which are situated adjacent to the C-Raf kinase domain.[140] Phosphorylation of these residues relieves the inhibitory effects of the regulatory domain on the kinase domain.[141] S338, which is the evolutionarily conserved PAK phosphorylation site that resides on the amino-terminal side of the kinase domain, is critical for Raf activation.[134-136,140,142] This site is also phosphorylated in response to stimulation by growth factors, integrins, Ras, PAK1, and PAK3.[78,136,143] The homologous site on B-Raf, S445, is constitutively phosphorylated, accounting for the higher basal activity of B-Raf. Ras presumably phosphorylates this site by activating PI3K. Activated mutants of Rac

and Cdc42 are also capable of inducing phosphorylation of S338 by activating PAK. Y341 is phosphorylated by the Src family of non-RTKs, Janus kinase, and erythropoietin.[85,92] The substitution of this tyrosine residue by aspartate in B-Raf may explain why B-Raf is fully inducible by Ras, whereas A-Raf and C-Raf require both Ras and Src for full activation.[124] However, Ras-mediated recruitment of C-Raf to the cell membrane and Src activation are not the only steps involved in the activation of C-Raf.

A-Raf, which is structurally similar to C-Raf, is activated in a similar manner; however, the pertinent structural and activational aspects of B-Raf differ from those of A-Raf and C-Raf. Although the structural domains and phosphorylation sites of Raf proteins differ, the greater degree of phosphorylated amino acids in B-Raf confers a 15- to 20-fold higher level of kinase activity in the basal state than either A-Raf or C-Raf, and B-Raf is therefore a much more robust activator of ERK phosphorylation.[85,144,145] The differential splicing of *B-raf* may also account for the distinct kinase activity of the protein. In addition, the structures of several B-Raf mutants mimic the conformational changes unique to phosphorylated wild-type B-Raf, which may explain the ability of B-Raf mutants to activate ERK in the absence of stimulation.

Other interactions. In addition to phosphorylation events, the activation status of C-Raf is regulated by protein-protein and protein-lipid interactions. As shown in Figure 2, C-Raf interacts with a diverse array of scaffolding proteins (kinase suppressor of Ras and MEK partner-1), adaptor proteins (Bcl-2–associated athanogene-1), chaperone proteins (Hsp90 and Hsp70), substrates (retinoblastoma protein [Rb]), lipids (phosphatidic acid, cholesterol-rich caveolae, and cytosolic lipid rafts), and cellular constituents, many of which, in turn, modulate its kinase activity.[95]

Activation of Downstream Effectors by Raf

Activated Raf principally propagates signaling by phosphorylating the two dual-specificity MAPKKs, MEK1 and MEK2 (also referred to as MKK1 and MKK2; Figs 1 and 2).[75] The Raf isoforms are the best characterized MEK1 and MEK2 activators, and all Raf isoforms activate MEK1, whereas only B-Raf and C-Raf activate MEK2. MEK1 and MEK2 contain a proline-rich sequence that enables recognition and activation by Raf.[125,146-153] This sequence, which is not present in other MAPKKs, may explain why Raf preferentially activates MEK1 and MEK2, whereas the dual-specificity $p38^{MAPK}$ kinases (MKK3 and MKK6) and JNK kinases (MKK4 and MKK7) phosphorylate $p38^{MAPK}$ and JNK, respectively. Although both A-Raf and C-Raf are capable of activating other signaling elements independent of MAPK pathway activation, such as nuclear factor-kappa B (NF-κB), Rb, and Bcl-2, MEK1 and MEK2 are the only known substrates for B-Raf.[154-157] A consistent theme in studies on MEK/ERK activation by Raf is that B-Raf is far more potent at activating downstream kinases than either A-Raf or C-Raf. Several lines of evidence also indicate that B-Raf has a much higher affinity for its substrate than the other Raf isoforms and is 50-fold more potent at phosphorylating MEK1 and MEK2 than either A-Raf or C-Raf.[125,158]

The respective downstream substrates of MEK1 and MEK2 are ERK1 ($p44^{MAPK}$) and ERK2 ($p42^{MAPK}$), which are translocated to the nucleus where they ultimately induce an array of cytoplasmic and nuclear regulatory proteins.[50,159-162] Effectors include the nuclear transcription factors Elk-1, Fos, Jun, AP-1, and Myc, which regulate genes encoding proteins that play key roles in proliferation, angiogenesis, metastasis, and resistance to anticancer therapeutics.[51] As a result, cell cycle regulators, such as cyclins D1 and E and Cdc activator 25 phosphatase, are positively regulated,[163,164] whereas $p27^{kip-1}$ and other inhibitors of cyclin-dependent kinases (cdk) are negatively regulated.[51] These actions favor progression through cell cycle checkpoints, aberrant growth, dedifferentiation, and cell survival.

C-Raf activates other cellular effectors, but the extent of the interdependence of these actions on MEK1 and MEK2 is not clear. For example, C-Raf activation regulates cytoskeleton formation by modulating the polymerization status of vimentin.[165] Cell survival signaling is also regulated by C-Raf, which induces phosphorylation of Iκ B in the NF-κB–Iκ B complex. This action releases activated NF-κB, which is then translocated to the nucleus where it mediates transcription of antiapoptotic factors.[155,166] Other antiapoptotic effects of C-Raf are mediated by a mitochondrial pool of the protein, which, on stimulation, localizes to the mitochondrial membrane where the protein interacts with and phosphorylates Bcl-2, Bcl-2–associated athanogene, and other pro-apoptotic regulators, abrogating their pro-apoptotic effects.[157,167] The antiapoptotic effects of C-Raf are also mediated through the ankyrin-repeat protein Tvl-1 and apoptosis signal-regulated kinase-1.[166,168,169] In addition, C-Raf phosphorylates Rb, p53, Cdc25, and other cell cycle regulatory proteins in metaphase.[156,170,171] Lastly, C-Raf induces transcription of the multidrug resistance gene *mdr-1*, and its activation has been associated with multidrug resistance.[172] In summary, Raf mediates essential cellular processes that signal proliferation, survival, and drug resistance.

RAF MUTATIONS IN HUMAN CANCER

General

Constitutively active mutant Raf proteins are predominately a result of point (missense) mutations, deletions, amplification, and rearrangements of *raf*.[173-176] Such genetic alterations have been identified in malignant melanoma, hematopoietic cancers, and cancers of the thyroid, breast, kidney, liver, larynx, biliary tract, and other organs, as shown in

Table 1.[173-176] Although initial efforts at identifying *raf* mutations in human cancer focused on *C-raf*, the advent of high-throughput gene sequencing led to the identification of activating *B-raf* mutations as the predominant genetic aberrations.[11,122,145,177]

B-raf *Mutations*

Recently, a sequence screen of 923 cancer samples for genes mutated in human cancers identified somatic mutations in a notable proportion of tumor samples.[9] Somatic *B-raf* mutations were demonstrated in 60% to 70% of malignant melanomas and in moderate to high rates in carcinomas of the colon, ovary, and thyroid (papillary), implicating activating oncogenic *B-raf* mutations as critical promoters of these malignancies.[9,14-17] Furthermore, somatic *B-raf* mutations were found, albeit at lower rates, in glioma, sarcoma, non-Hodgkin's lymphoma, acute myeloid leukemia, and carcinomas of the breast, lung, and liver. Interestingly, *C-raf* mutations were not identified in a series of 545 cancer samples, including melanomas and carcinomas of the colon, ovary, and lung.[9]

Sequence analysis of *B-raf* in human cancer has identified more than 30 single-site missense mutations, principally encoding amino acids in the kinase domain of B-Raf, whereas the constitutive activity and transforming potential of C-Raf result from loss of the auto-inhibitory amino-terminal region, as well as gene rearrangements.[6,175,176] Most *B-raf* mutations are caused by thymidine-to-adenine transversions at nucleotide position 1796 in exon 11 or 15, which encode a valine-to-glutamic acid substitution at amino acid 599 (^{V599E}B-Raf) in the activation segment (kinase domain) of the protein. Interestingly, structural changes in the activation segment as a result of the insertion of an acidic residue close to a site of regulated phosphorylation mimic phosphorylated B-Raf.[9] *^{V599E}B-Raf* possesses the hallmarks of a conventional oncogene because the kinase activity of its encoded protein is greatly elevated; it constitutively stimulates ERK in vivo in the absence of Ras activation; and it transforms NIH3T3 cells.[144] Furthermore, the basal kinase activity of ^{V599E}B-Raf is 12.5-fold higher than that of wild-type B-Raf, and its responsiveness to stimulation by oncogenic H-Ras is diminished. Furthermore, the transforming capacity of ^{V599E}B-Raf in NIH3T3 cells is 667-fold more efficient than that of wild-type B-Raf, whereas the equivalent mutation introduced into C-Raf (V492E) confers 10-fold lower kinase activity and transforming capacity.[85]

The discovery of *B-raf* mutations in 60% to 70% of malignant melanomas is surprising because early studies attributed the hyperactivation of the Raf/MEK/ERK module of the MAPK pathway in melanoma to an abundance of autocrine and paracrine growth factors. Interestingly, *B-raf* mutations are not found in uveal melanoma, which differs from cutaneous melanoma in that abnormalities of chromosome 6 are found only in uveal melanoma, suggesting that there are distinct pathways for melanoma formation.[178,179] Further studies evaluating the function of ^{V599E}B-Raf in benign and dysplastic nevi may yield important information about the type and timing of events required for tumorigenesis. Interestingly, the ^{V599E}B-Raf allele is found in as many as 80% of benign nevi, suggesting a role for oncogenic *B-raf* in nevus formation and melanoma initiation.[180] However, there is no direct evidence that benign nevi harboring ^{V599E}B-Raf progress to malignancy, and most cases may actually represent terminally differentiated lesions analogous to nondysplastic colorectal aberrant crypt foci that harbor *K-ras* mutations in the absence of *adenomatous polyposis coli* (*APC*) mutations. *APC* mutations are generally considered to be of low malignant potential, whereas *K-ras* mutations that arise after *APC* mutations promote colorectal tumor progression.[181,182] Further studies are also needed to determine whether the prevalence of *B-raf* mutations in melanoma relates to the site of the primary tumor, sun exposure, and radiation damage. Similar findings have been noted in the setting of papillary thyroid carcinoma, in which up to 69% of tumors harbor ^{V599E}B-Raf, whereas benign thyroid tumors and both follicular and medullary thyroid carcinomas do not.[18,19] It is notable that *B-raf* mutations are common in melanoma and thyroid cancers and that both melanocyte and thymocyte growth is positively regulated by cAMP. Interestingly, B-Raf is thought to be the key Raf isoform that transduces cAMP-dependent growth signals in both cell types, which may account for their vulnerability to transformation by activating mutations of this kinase.[183,184]

Analysis of other much less common oncogenic B-Raf mutants, most of which cluster adjacent to valine 599 or in the G loop ATP-binding region, suggest that the mutated proteins stimulate kinase activity in a manner similar to ^{V599E}B-Raf.[9] Nevertheless, it is intriguing that several of these mutations involve highly conserved or invariant residues in the catalytic domain, which are required by other kinases for optimal activity. This raises the question of how these mutants promote tumorigenesis.[8,9,185] It should also be noted that *B-raf* mutations outside the kinase domain have been identified, and other mutations will likely be identified as the gene is sequenced in other types of malignancies.[11]

Mutations of *B-raf* and *ras* are essentially mutually exclusive, implying that these genes belong to the oncogenic signaling pathway. Fewer than 1% of cancers with *B-raf* mutations have simultaneous *ras* mutations, and of the 1% that have mutations of both genes, the *B-raf* mutations are almost never ^{V599E}B-Raf.[8,9,14] In colorectal carcinoma, both genes are mutated at high frequencies in the same types of premalignant lesions and at the same stages in the transition from adenoma to carcinoma.[8,14] A strong association exists between mismatch repair deficiency and the presence of the mutant ^{V599E}B-Raf protein in colorectal carcinoma, which

may be a result of the underlying DNA repair defect.[14] Further reflecting the redundancy of the MAPK pathway, a high fraction of papillary thyroid cancers harbor either ^{V599E}*B-Raf*, mutant *K-ras*, or mutant *RET*.[9,20] Harboring more than one mutation is quite rare, although a moderate fraction of low-grade ovarian tumors harbor either ^{V599E}*B-Raf* or mutant *K-ras*.[9,20] This finding may represent a unique paradigm of human tumorigenesis through mutations of these signaling proteins that lie in tandem.[8,9] However, concomitant *ras* mutations have been identified in cancers that harbor uncommon *B-raf* mutations in the G loop region, suggesting that there may be differences in molecular pathways used by distinct mutant B-Raf proteins.[9]

C-raf *Mutations*

In contrast to *B-raf* mutations, no underlying genetic mechanisms predominate in human cancers that harbor *C-raf* mutations. Several types of genetic alterations, particularly gene rearrangements, have been demonstrated in human cancers sampled from patients with non–small-cell lung carcinoma and T-cell lymphoma harboring *C-raf* mutations.[10] In addition, constitutively active C-Raf has been associated with site-specific *C-raf* mutations, and a structurally aberrant C-Raf protein that is truncated in its amino-terminal regulatory domain has been identified in tumor samples from patients with carcinomas (kidney, lung [small cell], liver, and pancreas), sarcomas (soft tissue and bone), and CNS malignancies (glioma, glioblastoma, and ependymoma).[6,186,187] However, neither specific genetic nor structural aberrations have been identified in a sizeable proportion of human cancers in which C-Raf is activated in the absence of upstream Ras activation.[6,175,177]

THERAPEUTIC STRATEGIES TARGETING RAF

Given the high proportion of cancers with constitutively activated Raf, Ras mutations, or growth factor hyperactivity, which result in increased signaling through Raf, Raf is an ideal target for therapeutic development. Although there have been many attempts to develop therapeutics against Raf, most efforts have been directed at C-Raf rather than B-Raf. To decrease Raf production and inhibit its activation, antisense oligonucleotides (ASONs), small-molecule kinase inhibitors, and dominant interfering DNA constructs are being developed. In addition, other therapeutics that indirectly target Raf include inhibitors of chaperone proteins (eg, geldanamycin analogs), which destabilize Raf, and histone deacetylase inhibitors, which reduces *raf* expression.[188]

ISIS 5132 (CGP 69846A): AN ASON INHIBITOR OF C-RAF

The specificity of nucleotide base pairing provides the rationale for using ASONs as therapeutics against Raf.[189,190] This approach relies on the intracellular uptake of short synthetic ASONs that are complementary to Raf mRNA by mechanisms that have not been clearly elucidated. The ASON then hybridizes with its cognate mRNA, leading to RNAase H–mediated degradation of the complex. Alternatively, the ASON can sterically inhibit translation, which reduces synthesis of the encoded protein.

ISIS 5132 (CGP 69846A; ISIS Pharmaceuticals Inc, Carlsbad, CA) is a 20-base phosphorothioate ASON designed to hybridize to the 3′ untranslated sequence of *C-raf*.[191] Binding induces degradation of the C-Raf mRNA, which, in turn, decreases synthesis of C-Raf in a concentration-dependent manner.[192] The 50% inhibitory concentration (IC_{50}) value for both tumor proliferation and C-Raf expression is approximately 100 nmol/L.[192] Furthermore, treatment of mice bearing human lung and breast cancer xenografts produces impressive decrements in C-Raf, as well as antitumor activity.[191] In other models, ISIS 5132 decreases C-Raf expression and enhances sensitivity to both cytotoxics and radiation.[193] The phosphorothioate backbone of ISIS 5132 was engineered to confer resistance to digestive nucleases, which is manifested by plasma half-life values ranging from 30 to 85 minutes and extensive tissue distribution in mice.[194-196]

The feasibility of administering ISIS 5132 was explored in patients with advanced solid neoplasms on the following schedules: (1) 21-day continuous intravenous (IV) infusion (CIVI) every 28 days; (2) 2-hour IV infusion thrice weekly for 3 weeks every 28 days; and (3) 24-hour IV infusion weekly for 3 weeks every 28 days.[197-199] The principal toxicities were fever and malaise. Thrombocytopenia and anemia, which were typically moderate in severity, brief, and not cumulative, were also noted. Transient prolongation of the activated partial thromboplastin time and activation of the alternate complement pathway, which have been attributed to the phosphorothioate backbone of ISIS 5132, occurred in a dose-dependent manner. Dose-dependent elevations of the compliment component C3a, but not Bb or C5a, were noted. Although maximum tolerated doses were not clearly defined in the first two studies, plasma concentrations of intact ISIS 5132 achieved at the highest doses (6 and 4 mg/kg/d) exceeded IC_{50} values derived in vitro and were known to activate the alternate complement pathway in monkeys.[198] In the third study, an unacceptably high incidence of intolerable toxicities, particularly Coombs hemolytic anemia and acute renal insufficiency, was noted in patients treated at doses greater than 24 mg/kg/wk. The toxicities of ISIS 5132 were similar to those of other ASONs and, therefore, should not be interpreted as being related to target inhibition. Although several patients experienced protracted periods of stable disease, major tumor regression did not occur. *C-raf* mRNA levels in peripheral-blood mononuclear cells were consistently suppressed in patients receiving ISIS 5132 as a 2-hour IV

infusion thrice weekly for 3 weeks, but suppression of *C-raf* mRNA was not detected on the schedule of 24-hour CIVI weekly for 3 weeks every 28 days and not evaluated in the study of ISIS 5132 as a 21-day CIVI.

The antitumor activity of ISIS 5132 was evaluated in phase II studies in patients with advanced colorectal (15 patients, no prior treatment for metastatic disease), hormone-refractory prostate (16 patients, no prior chemotherapy), ovarian (22 patients, one to two prior systemic therapies), small-cell lung (four patients, one prior therapy), and non–small-cell lung (18 patients, no prior therapies) carcinomas.[200-203] Stable disease lasting 2.5 to 5.5 months was the best response in a sizeable proportion of patients, but there were no major tumor regressions. Nonetheless, these disappointing results should not diminish the potential importance of Raf as a therapeutic target because several alternative hypotheses, including the lack of validation of ASON technology as a platform that can confer robust anticancer activity and lack of documentation of *raf* mutational status in these clinical studies, may explain these results.

Small-Molecule Inhibitors of Raf Kinase

The identification of nearly 500 kinases that can be classified into at least 20 families based on structural homology and recent successes with kinase inhibitors have produced bountiful opportunities for small-molecule inhibitors of Raf kinase.[185] The elucidation of the crystalline structure of the ATP-binding domain of Raf has even further brightened these prospects.[204,205] Several classes of small molecules are currently being optimized from both mechanistic and pharmaceutical standpoints. In addition to blocking Raf kinase, small molecules directed at Raf also inhibit a wide range of other kinases by virtue of structural homology between the kinase families. Although it may be desirable for small-molecule therapeutics to impart inhibitory effects on multiple critical signaling pathways, these multifunctional aspects may also impart greater toxicity. Of the small-molecule Raf inhibitors in development, sorafenib (BAY 43-9006; Bayer Corporation Pharmaceutical Division, New Haven, CT; and Onyx Pharmaceuticals, Inc, Richmond, CA; Fig 5) is the furthest along.

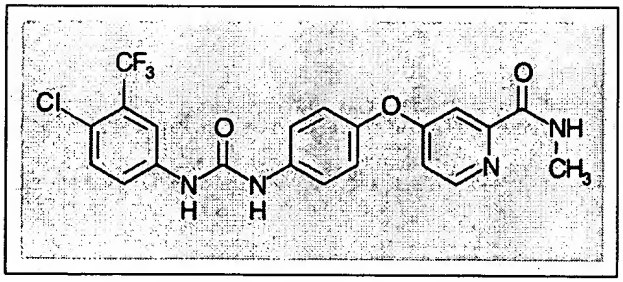

Fig 5. Chemical structure of sorafenib (BAY 43-9006).

Sorafenib (BAY 43-9006): Mechanism of Action and Preclinical Results

The bi-aryl urea sorafenib (4-{4-[3-(4-chloro-3-trifluoromethyl-phenyl)-ureido]-phenoxyl}-pyridine-2-carboxylic acidmethylamide-4-methylbenzene-sulfonate; BAY 43-9006) is the first molecule of its class to undergo clinical development. Originally identified by high-throughput screening of small molecules against C-Raf kinase, sorafenib was found to be a potent competitive inhibitor of ATP binding in the catalytic domains of C-Raf, wild-type B-Raf, and ^{V599E}B-Raf mutant. As shown in Table 2, the IC_{50} values of sorafenib against C-Raf, wild-type B-Raf, and the ^{V599E}B-Raf mutant in a biochemical assay are in the low nanomolar range, whereas sorafenib does not inhibit MEK1, ERK1, erbB1, or erbB2.[206] Sorafenib potently inhibited activation of the MAPK pathway and ERK phosphorylation in human cancer cell lines, irrespective of whether they harbored *K-ras* mutations, ^{V599E}*B-Raf*, or both.[207] Further characterization of sorafenib in biochemical assays showed potent inhibition of pertinent RTKs involved in tumor progression and angiogenesis, including human and murine vascular endothelial growth factor receptor (VEGFR) -2, VEGFR-3, platelet-derived growth factor receptor-beta (PDGFR-β), Flt-3, c-Kit, p38α, and fibroblast growth factor receptor-1 (Table 2). In contrast, erbB1, insulin-like growth factor-1, c-met, and erbB2 RTKs were not inhibited. The kinase activities of PKA, PKB, PKCα, PKCγ, cdk1/cyclin B, and pim-1 were also insensitive.[208] Furthermore, sorafenib inhibited various nonkinase

Table 2. Biochemical Kinase Selectivity Profile for Sorafenib (BAY 43-9006)[206]

Biochemical Assay	IC_{50} (nmol/L)
C-Raf	6
B-Raf, wild-type	22
B-Raf mutant, V599E	38
VEGFR2	90
mVEGFR-2	6
mVEGFR-3	12
mPDGFR-β	57
Flt-3	58
c-Kit	68
p38α	38
FGFR-1	580
EGFR, HER-2, ERK1, MEK1, IGFR-1, c-met, c-yes, PKB, PKA, cdk1/cyclin B, PKCα, PKCγ, pim-1	> 10,000

Abbreviations: cdk, cyclin-dependent kinase; EGFR, epidermal growth factor receptor; ERK, extracellular signal–regulated kinase; FGFR-1, fibroblast growth factor receptor-1; HER-2, human epidermal growth factor receptor 2; IC_{50}, concentration of sorafenib that inhibits the kinase activity by 50%; IGFR, insulin-like growth factor receptor; mPDGFR, mouse platelet-derived growth factor receptor; PKA, protein kinase A; PKB, protein kinase B; PKC, protein kinase C; m, murine; MEK1, mitogen-activated protein kinase kinase 1; VEGFR, vascular endothelial growth factor receptor.

targets, including adenosine A3, dopamine D1, and muscarine M3, albeit at much higher (micromolar) concentrations than kinase targets.[209] In cellular assays, sorafenib reduced basal phosphorylation of the MAPK pathway in a panel of human breast, melanoma, pancreatic, and colon cancer cell lines expressing either mutant K-Ras, mutant B-Raf, or wild-type Ras or Raf. Interestingly, several non–small-cell lung cancer cell lines expressing mutant *K-ras* were resistant, presumably because Raf-independent activation of MEK is operative in these cells.[208,210] In other cell-based assays, sorafenib inhibited phosphorylation of several pro-angiogenic RTKs, including both human VEGFR-2 and murine VEGFR-2, murine VEGFR-3, PDGFR-β, and Flt-3.[208,210] In nude mice bearing human xenografts derived from melanoma and colon, pancreas, breast, and lung carcinomas, sorafenib treatment resulted in a high level of tumor growth inhibition without appreciable toxicity.[206] Most early evaluations used the HCT116 human colon xenograft because its tumorigenicity depends on K-Ras activation. Extending the duration of sorafenib treatment attained protracted antitumor efficacy, even when treatment was initiated in settings of high tumor burden. Significant growth inhibition was noted after treatment of well-established human xenografts with *B-raf* mutations (HT-29, Colo205, and DLD-1 colon), *K-ras* mutations (NIH-H460 and A459 lung; MiaPaCa pancreas), and both *K-ras* and *B-raf* mutations (MDA-MB-231 breast) with sorafenib at doses of 7.5 to 60 mg/kg daily for 9 days. *B-raf*–mutated MDA-MB-231 breast cancer xenografts, which were reduced in size by 42%, on average, after only 9 days of treatment with 30 mg/kg of sorafenib, were the most sensitive.[208,210] Relevant activity against the human SKOV-3 ovarian xenograft that harbors wild-type Ras but overexpresses both erbB1 and erbB2 was also noted.

Concurrent with the demonstration that sorafenib is efficacious in a molecularly diverse range of human tumor xenografts, translational studies have demonstrated inhibition of the MAPK pathway after 5 days of sorafenib treatment of HT-29, DLD-1, HCT-116, and MDA-MB-231, but not Colo-205 xenografts. In the Colo-205 tumors, in which concurrent assessments of vascular effects were performed, tumor neovascularization was reduced dramatically. The cumulative results of these studies suggest that sorafenib inhibits tumor progression by blocking cellular proliferation that is dependent on activation of the MAPK pathway and/or inhibiting tumor angiogenesis through VEGFR-2, VEGFR-3, and/or PDGFR-β. Recent studies suggest C-Raf inhibition may also promote the death of endothelial cells as a result of their specific requirements for stimulation by VEGFR-2.[211] The results indicate that sorafenib is efficacious not only against human tumors with *ras* and/or *raf* mutations, but also against tumors that overexpress growth factor receptors that signal through Ras and the Raf/MEK/ERK module. However, it is important to note that the relative potency of sorafenib against various kinases, particularly VEGF, must be considered in assessing the value of sorafenib as a Raf kinase inhibitor, as well as in drawing conclusions about the value of Raf kinase as a molecular target against cancer.

Favorable cytotoxic effects were noted after treatment of a broad spectrum of human cancer cell lines and xenografts harboring both wild-type and mutated forms of *ras* or *raf* with sorafenib and either fluorouracil, paclitaxel, gemcitabine, gefitinib, vinorelbine, doxorubicin, irinotecan, or its active SN-38 metabolite.[212] Treatment of human tumor xenografts with sorafenib plus paclitaxel, irinotecan, gemcitabine, or cisplatin did not enhance the toxicity or diminish the activities of the therapeutics.

Pharmacokinetic studies in rodents and dogs have demonstrated that sorafenib clearance is much lower than normal liver plasma flow. Its low steady-state volume of distribution (approximately 0.7 to 0.93 L/kg) suggests that tissue affinity is low and plasma protein binding is high (mean free fraction, 1.2% [human] to 2.5% [mouse]). The pharmacokinetics in mice are dose proportional over a biologically relevant dosing range, and tissue concentrations are several fold higher than IC_{50} values in vitro.[206,208,213] At higher doses, drug exposure increases disproportionately, possibly because of saturation of gastrointestinal absorption. Autoradiographic studies have revealed homogeneous drug distribution to peripheral tissues and modest penetration across the blood-brain barrier. The mean terminal half-life ranges from 6 to 7 hours. In rodents, oral bioavailability is high (approximately 79%). Drug disposition is principally by CYP3A4 metabolism, followed by biliary and fecal excretion (approximately 90%). CYP1A, CYP2C9, CYP2C19, and CYP3A are not induced after incubating drug with microsomal extracts from human hepatocytes. However, in vitro metabolism studies in human systems indicate extensive metabolism by CYP3A, and early clinical data indicate that disposition is principally by hepatic metabolism and fecal excretion. Sorafenib is a modest inhibitor of CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4, and the propensity for interactions between sorafenib and drugs that inhibit and induce P-450 systems exists.

In rodents and dogs, sorafenib is well tolerated. Principal toxicities include emesis, diarrhea, and transaminase elevations. Histopathologic studies have revealed dose-related degenerative changes in the liver, stomach, duodenum, pancreas, kidneys, heart, testes, and ovaries and regenerative changes in the liver, pancreas, duodenum, and kidneys. Hypocellularity and necrosis of hematopoietic and lymphoid tissues and unusual findings involving the teeth and growth plate of the femur have been noted.

Clinical Evaluations

Phase I studies. Phase I end points were evaluated in patients with advanced solid malignancies in studies of the

following daily oral schedules: (1) 7 days every 15 days; (2) 21 days every 28 days; (3) 28 days every 35 days; and (4) continuous treatment. The principal dose-related toxicities were diarrhea, vomiting, skin rash, fatigue, hypertension, and palmar-plantar erythrodysesthesia (hand-foot syndrome). Hand-foot syndrome was characterized by desquamation and discomfort of the digits, all of which were reversible. Clinically relevant elevations in serum amylase and lipase and both lymphopenia and anemia were uncommon. The incidences of intolerable toxicities, particularly diarrhea and hand-foot syndrome, were unacceptably high at sorafenib doses exceeding 400 mg twice daily on a continuous schedule, which was recommended for phase II trials. Tumor regression was noted on several schedules, particularly when doses exceeded 200 mg twice daily. One patient each with hepatocellular carcinoma and renal cell carcinoma (RCC) had partial responses, whereas tumor regressions of lesser magnitude occurred in patients with RCC and colorectal and ovarian carcinomas. Furthermore, approximately 50% of patients with colorectal, ovarian, hepatocellular, renal, and breast carcinomas had stable disease as their best response.[214] Pharmacokinetic studies revealed dose proportionality up to 600 mg twice daily and high interpatient variability. Steady-state was achieved by 7 days, and terminal half-life values ranged from 30 to 45 hours. ERK1/2 phosphorylation in $CD7^+$ peripheral-blood mononuclear cells was inhibited.[215,216]

Disease-directed studies. The principal paradigm adopted for disease-directed evaluations of sorafenib represents a radical departure from traditional phase II approaches. Although phase II studies are being performed in malignancies of high interest, the principal disease-directed evaluation strategy was a randomized discontinuation trial. This unorthodox approach was undertaken because the predominant clinical benefit of the agent, particularly in patients whose tumors were not screened for molecular aberrations known to increase the probability of responding, was projected to be increased progression-free survival (PFS), which was also the principal beneficial effect in preclinical studies. In addition, because sorafenib inhibits multiple kinases, the use of any empiric screening and/or enrichment strategy, as well as any particular malignancy, could produce false-negative results. In contrast to randomized phase II studies, which lack sufficient statistical power to discern small to moderate, albeit relevant, differences between treatments, the randomized discontinuation study is designed so that there is an initial process of natural enrichment of the study population with patients who may have experienced benefit to treatment before patients are randomly assigned to either continue or discontinue drug treatment.[217]

The randomized discontinuation study is felt to be ideal for sorafenib and agents whose main benefit is expected to be tumor growth delay, which is not readily detected in nonrandomized studies. At the end of an initial lead-in phase, in which all patients receive the study drug, patients who experience a relevant degree of tumor growth are removed from the study. This weeding out process enriches the study population with patients who will most likely benefit from further treatment, thereby increasing the probability that the randomization step will be more efficient at detecting tumor growth inhibition related to drug. In essence, the lead-in period may furnish data about the inherent potential of the agent to induce tumor regression and can suffice as multiple phase II studies, each of which can be sized in real time to provide a requisite level of statistical power. At the end of the lead in period, patients whose tumors have not progressed are randomly assigned to either continue or discontinue treatment, ideally in a double-blinded, placebo-controlled fashion. The natural selection or enrichment of the population before random assignment increases the efficiency of the trial, with as few as 20% of the standard number of randomly assigned patients. Nonetheless, a shortcoming of this approach relates to its inability to precisely quantify the magnitude of antitumor activity. However, if there is a clear difference in PFS between the randomly assigned arms, conclusions can still be generated about the general activity of the agent. Nevertheless, if the results meet a sufficient level of interest, resource-intensive phase III studies may ensue.

The randomized discontinuation study, as depicted in Figure 6, was designed to discern differences in PFS between patients treated with either sorafenib or placebo in the randomization period. The randomization stage was sized to discern PFS in patients with colorectal carcinoma, which frequently harbors *ras* mutations, although patients with many tumor types were enrolled. At the end of the 12-week period, in which all patients received sorafenib 400 mg twice daily, patients whose target lesions had increased in excess of 25% were taken off study. Because of concerns about randomly assigning patients who had potentially benefited from treatment, patients whose target lesions had regressed by greater than 25% were not randomly assigned and, instead, continued treatment until disease progression. Patients who experienced neither objective benefit of this magnitude nor disease progression were randomly assigned to either continue treatment with sorafenib or placebo. Because PFS was the primary end point in the randomization phase, placebo-treated patients who experienced progressive disease could be re-treated with sorafenib.

Patient accrual ended in January 2004, with 484 patients accrued at a rate of 36 patients per month at only five institutions in the United States and Europe. Of these, 408 patients were the focus of a recent report.[218] Tumor regression was noted in previously treated patients with advanced melanoma, sarcoma, RCC, and colorectal, thyroid, and pancreatic cancers. Most colorectal carcinoma patients treated with sorafenib developed disease progression before random assignment. RCC emerged as a central focus of the

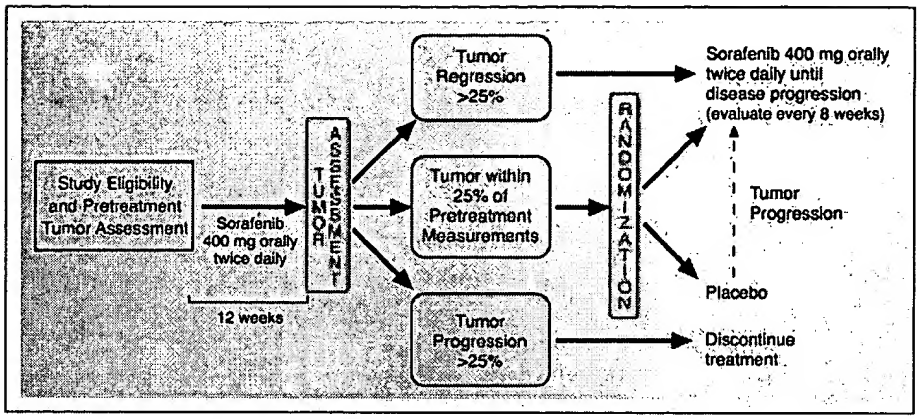

Fig 6. Schema of the randomized discontinuation trial with sorafenib (BAY 43-9006).

study, and the accrual targets for RCC were reset higher as the activity of sorafenib in RCC became increasingly evident. In the run-in phase, 202 patients with RCC were treated with sorafenib and were the focus of a recent report in May 2005.[219] At 12 weeks, 144 patients (71%) experienced tumor shrinkage or disease stabilization. Independently confirmed tumor shrinkage of ≥ 25% (partial response) was noted in eight (4%) of these patients. A total of 65 patients were entered onto the randomization phase, of whom 32 were treated with sorafenib and the rest received placebo. Sixteen patients (50%) in the sorafenib arm were progression free at 12 weeks postrandomization, compared with six patients (18%) in the blacebo arm (P = .0077). The median progression-free survival from randomization in the two arms was 24 weeks and 6 weeks, respectively (P = .0087). The unique design of this study served the broad mechanism of action of this promiscuous tyrosine kinase inhibitor, providing a sufficient level of flexibility to evaluate a wide range of end points and tumor types. The intriguing results in patients with RCC provided the basis for a phase III study, in which 800 patients with unresectable and/or measurable RCC who have received at least one prior systemic therapy are being randomly assigned to treatment with either sorafenib or placebo. The primary and secondary end points were overall survival and progression free survival, respectively. A total of 905 patients were randomly assigned, of whom 769 patients were the focus of a recent report.[220] Three hundred eighty-four patients were randomly assigned to the sorafenib arm, and the rest to the placebo arm. An independent response assessment was performed in 574 patients. Seven partial responses (2%) were noted in the sorafenib arm compared with none in the placebo arm. Disease stabilization was seen in 261 (78%) versus 186 (55%) patients in the sorafenib and placebo arms, respectively, and disease progression was noted in 29 (9%) versus 102 (30%) patients, respectively. The median progression-free survival for patients in the sorafenib arm was 24 weeks, compared with 12 weeks in the placebo arm (hazard ratio = 0.44; P = .000001). The substantial benefit due to sorafenib was apparent across all patient subgroups. Because of the magnitude of the benefit noted upon analysis, treatment unblinding was performed, and patients randomly assigned to placebo were allowed to cross over to sorafenib treatment.

There was considerable interest in the melanoma patients who participated in the randomized discontinuation study based on the high incidence of *B-raf* mutations in melanoma.[218] In the June 2004 report that focused on the first 20 patients enrolled, five patients developed cutaneous toxicity of grade 3 severity, and two patients developed hypertension that required intervention.[221] Of 19 patients whose disease had been evaluated, 15 patients developed progressive disease before or at the planned 12-week assessment, whereas one patient had a partial response, and three patients had stable disease. Although a complete survey of *B-raf* mutations was not available for the June 2004 report, the negligible antitumor activity in a malignancy with a 60% to 70% incidence of constitutive *B-raf* mutations implies that sorafenib alone on the dose schedule evaluated lacks sufficient activity at inhibiting B-Raf kinase. Phase II studies are also ongoing in advanced hepatocellular and non–small-cell lung carcinomas and other malignancies.

Combination studies. The feasibility of administering sorafenib with various other agents is being evaluated in early clinical evaluations. Flaherty et al,[222] who conducted a phase I study of sorafenib plus carboplatin and paclitaxel and then focused on the activity of the regimen in patients with melanoma who were enrolled at the maximum-tolerated dose, have provided some of the most intriguing results. Both untreated and previously treated patients with progressive growth of solid neoplasms before study enrollment were treated with fixed doses of carboplatin area under the curve 6 and paclitaxel 225 mg/m^2 IV over 3 hours on day 1 followed by sorafenib at doses of 100, 200, or 400 mg twice daily from days 2 to 19 of a 3-week course. All dose iterations were well tolerated, and toxicity rates did not exceed those expected with carboplatin and paclitaxel in the absence of sorafenib. Sixty-seven patients with melanoma,

most of whom were enrolled onto the phase II stage, have been treated. Of the 35 assessable patients with melanoma at the time of the report, 14 patients (40%) had partial responses, all of which lasted for at least 6 months, and 15 patients (43%) had stable disease as the best response. The median PFS time was estimated at 8.4 months. Of 25 patients whose mutational status was assessed, 15 (60%) had ^{V599E}*B-Raf*, but the propensity to respond did not depend on *B-Raf* mutational status. Five (33%) of 15 patients with ^{V599E}*B-Raf* and six (60%) of 10 patients with wild-type B-Raf had partial responses. These results are impressive in light of the negligible activity of sorafenib as a single agent in patients with melanoma, implying that sorafenib may operate principally by inhibiting VEGF RTKs, perhaps by enhancing the penetration of cytotoxics into tumors and/or decreasing intratumoral pressure. A phase III study evaluating the paclitaxel-carboplatin regimen with and without sorafenib is planned.

In a phase I study of sorafenib and gemcitabine, the maximum-tolerated doses were gemcitabine 1,000 mg/m^2 IV weekly for 7 of every 8 weeks, followed by weekly for 3 of every 4 weeks and sorafenib 400 mg twice daily.[223] Of 19 previously treated patients in the phase I study, two patients with ovarian carcinoma had partial responses; whereas one (4.3%) of 23 previously untreated subjects treated in an expanded stage at the recommended dose had a partial response.[223] No pharmacokinetic interactions were evident. Similarly, regimens consisting of sorafenib plus doxorubicin, irinotecan, or oxaliplatin were well tolerated, and pharmacokinetic interactions were not apparent.[224-226] In a phase I study of sorafenib and doxorubicin, the principal toxicities were neutropenia and hand-foot syndrome, which did not preclude administering each agent at relevant doses (sorafenib 400 mg twice daily and doxorubicin 60 mg/m^2 IV every 3 weeks).[222] One patient with mesothelioma had a partial response, and 17 patients (52%) had stable disease. Of note, four patients with hepatocellular carcinoma experienced stable disease lasting at least 12 months. Relevant doses of sorafenib (400 mg twice daily) and oxaliplatin (130 mg/m^2 every 3 weeks) were well tolerated.[227] Two patients with gastric carcinoma had partial responses lasting 14 and 21 weeks, and eight (42%) of 19 assessable patients had stable disease. Pharmacokinetic interactions were not evident. The activity of these agents in patients with colorectal carcinoma and the feasibility of administering sorafenib with other therapeutics are being studied.

Other Phrarmacologic Inhibitors of Raf Kinase

Besides sorefanib, other small-molecule competitive inhibitors of the ATP-binding site of Raf proteins have been developed. L-779450 (Merck Pharmaceuticals Inc, Nutley, NJ), a competitive inhibitor of the ATP-binding site of C-Raf, has demonstrated activity in the nanomolar range against C-Raf–overexpressing human tumors in vitro and is much more effective at inhibiting the kinase activity of C-Raf and A-Raf than B-Raf.[228,229] Likewise, the phenol substituted oxindole derivative SB203580 (GlaxoSmithKline Pharmaceuticals, Philadelphia, PA) inhibits C-Raf kinase in the low nanomolar range, but micromolar concentrations are required to inhibit ERK phosphorylation in cell culture.[230,231] Interestingly, SB203580 may paradoxically activate C-Raf through an autocrine feedback loop as a result of MEK inhibition.[232] Although C-Raf is a weak kinase, the significance of this observation is not known.

The tumor-inhibitory and cytotoxic effects of naturally occurring ansamycin antibiotics, particularly geldanamycin analogs that bind to Hsp90 and destabilize HSP90-dependent proteins, produce impressive decrements in C-Raf expression.[233] However, the effects of these agents on C-Raf may be nonspecific because Hsp90 plays a critical role in stabilizing and conferring functionality to a wide array of important cellular proteins such as v-Src, EGFR, HER-2/*neu*, cdk4, Akt2, and mutated p53, all of which are inhibited by the abrogation of Hsp90.[233] Thus, the growth inhibitory and pro-apoptotic effects of geldanamycin analogs, such as 17-allylamino-17-demethoxygeldanamycin, may not be a result of direct effects on C-Raf alone, considering the pleiotropic effect of these compounds.[234] Radicicol, a novel macrocyclic antibiotic isolated from the fungus *Monosporium bonorden* and potent inhibitor of Hsp90, has exhibited impressive tumor growth–inhibitory activity against a wide range of human tumor cell lines and xenografts, and both its antitumor and pharmaceutical properties are being optimized.[233] Radicicol may, in part, inhibit tumor growth by destabilizing and depleting C-Raf.[235] The *O*-carbamoylmethyloxime derivatives may be superior to radicicol from both mechanistic and pharmaceutical perspectives.[235]

Dominant Interfering DNA Constructs

Dominant interfering DNA constructs that specifically target tumor cells with anti-*raf* genes have been described. One such method, which involves coupling a cationic lipid-based nanoparticle to an $\alpha v\beta 3$ integrin ligand, seems to deliver genes to newly developing blood vessels of tumors that arise in mice after injection of melanoma cells. Hypothetically, by coupling a cDNA-encoding kinase-inactive C-Raf to the nanoparticle, this dominant negative version of C-Raf is delivered to the neovasculature of developing tumors and induces apoptosis and tumor regression. Paradoxically, the inhibition of C-Raf may induce tumor regression not by affecting MEK/ERK activation, but by inhibiting the MEK kinase–independent role of C-Raf in promoting tumor survival.[236] It is certainly possible that other therapeutics directed against C-Raf act in a similar fashion.

CONCLUSION

It is hopeful that therapeutics designed based on understanding the primary molecular defects governing malignant cell proliferation will be more efficacious and less toxic than nonspecific cytotoxics. It is clear that aberrant forms of Raf are the principal drivers of many types of cancer. Furthermore, the potential therapeutic benefit conferred by therapeutics targeting Raf may not be limited to malignancies with mutant Raf because some cancers with wild-type Raf associated with growth factor receptor hyperactivity and/or Ras mutations are extraordinarily sensitive to Raf inhibition.

The knowledge that some tumors have driving target aberrations coupled with gene-sequencing data has provided the means to establish proof of principle about the validity of targets and/or targeted therapeutics. For Raf, rational patient enrichment strategies based on the presence of specific aberrations of Raf, Ras, and growth factor receptors can be formulated from the outset and dynamically optimized in the course of development. This is not to say that therapeutics targeting Raf will ultimately be restricted to niche indications because molecular aberrations are shared by many cancers. Instead, after proof of principle in cancers that are solely driven by a target aberration, such as melanoma and pancreatic carcinoma in the case of Raf and Ras, respectively, studies could then be conducted in tumors in which the target contributes to, but may not be the sole driver of, tumor growth. Although tumor regression rates may be negligible in these settings, the principal therapeutic effects of target inhibition in tumors with multiple contributory molecular aberrations may be best appreciated in randomized trials designed to detect differences in overall survival, PFS, and other end points that reflect tumor growth inhibition.

Although the early results with sorafenib have been encouraging, emerging clinical data do not irrefutably validate Raf as its relevant target. Instead, the intriguing activity of sorafenib in RCC, its lack of robust single-agent activity in melanoma, and its ability to enhance the activity of chemotherapeutics may reflect its greater potency at inhibiting VEGFR or other, as of yet unidentified, RTKs. To this end, the lack of robust activity with ASONs targeting Raf should not negate the importance of Raf as a therapeutic target because mutations of *Raf* and *Ras* were not assessed in studies of these agents and there are many unanswered questions about the validity of antisense strategies as therapeutic platforms. Although knowing the precise mechanism of sorafenib's antitumor activity may make little difference with regard to its ultimate utility, such information may be used to optimize the therapeutic indices of the next generation of therapeutics targeting Raf. In concert with clinical evaluations, *Ras/Raf* gene sequencing studies and assessments of relevant biologic markers may facilitate these efforts. Most importantly, the role of Raf in driving tumor proliferation must be further understood. Issues pertaining to the structural and functional basis of Raf and Ras mutations and their interrelationships and roles in tumorigenesis, proliferation, and cell survival must be addressed to develop more effective therapeutics against Raf and related targets.

Authors' Disclosures of Potential Conflicts of Interest

Although all authors completed the disclosure declaration, the following authors or their immediate family members indicated a financial interest. No conflict exists for drugs or devices used in a study if they are not being evaluated as part of the investigation. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

Authors	Employment	Leadership	Consultant	Stock	Honoraria	Research Funds	Testimony	Other
Amita Patnaik			Genentech (A)					
Eric K. Rowinsky						Bayer (C)		
Dollar Amount Codes	(A) < $10,000	(B) $10,000-99,999	(C) ≥ $100,000	(N/R) Not Required				

REFERENCES

1. Khosravi-Far R, Der CJ: The Ras signal transduction pathway. Cancer Metastasis Rev 13:67-89, 1994
2. Campbell SL, Khosravi-Far R, Rossman KL, et al: Increasing complexity of Ras signaling. Oncogene 17:1395-1413, 1998
3. Vojtek AB, Der CJ: Increasing complexity of the Ras signaling pathway. J Biol Chem 273: 19925-19928, 1998
4. Chong H, Vikis HG, Guan KL: Mechanisms of regulating the Raf kinase family. Cell Signal 15:463-469, 2003
5. Joneson T, Bar-Sagi D: Ras effectors and their role in mitogenesis and oncogenesis. J Mol Med 75:587-593, 1997
6. Heidecker G, Huleihel M, Cleveland JL, et al: Mutational activation of c-raf-1 and definition of the minimal transforming sequence. Mol Cell Biol 10:2503-2512, 1990
7. Schaap D, van der Wal J, Howe LR, et al: A dominant-negative mutant of raf blocks mitogen-activated protein kinase activation by growth factors and oncogenic p21ras. J Biol Chem 268: 20232-20236, 1993
8. Yuen ST, Davies H, Chan TL, et al: Similarity of the phenotypic patterns associated with BRAF and KRAS mutations in colorectal neoplasia. Cancer Res 62:6451-6455, 2002
9. Davies H, Bignell GR, Cox C, et al: Mutations of the BRAF gene in human cancer. Nature 417:949-954, 2002
10. Storm SM, Rapp UR: Oncogene activation: C-raf-1 gene mutations in experimental and naturally occurring tumors. Toxicol Lett 67:201-210, 1993
11. Mercer KE, Pritchard CA: Raf proteins and cancer: B-Raf is identified as a mutational target. Biochim Biophys Acta 1653:25-40, 2003
12. Sommerer F, Vieth M, Markwarth A, et al: Mutations of BRAF and KRAS2 in the development

of Barrett's adenocarcinoma. Oncogene 23:554-558, 2004

13. Callans LS, Naama H, Khandelwal M, et al: Raf-1 protein expression in human breast cancer cells. Ann Surg Oncol 2:38-42, 1995

14. Rajagopalan H, Bardelli A, Lengauer C, et al: Tumorigenesis: RAF/RAS oncogenes and mismatch-repair status. Nature 418:934, 2002

15. Nikiforova MN, Kimura ET, Gandhi M, et al: BRAF mutations in thyroid tumors are restricted to papillary carcinomas and anaplastic or poorly differentiated carcinomas arising from papillary carcinomas. J Clin Endocrinol Metab 88:5399-5404, 2003

16. Kimura ET, Nikiforova MN, Zhu Z, et al: High prevalence of BRAF mutations in thyroid cancer: Genetic evidence for constitutive activation of the RET/PTC-RAS-BRAF signaling pathway in papillary thyroid carcinoma. Cancer Res 63:1454-1457, 2003

17. Pollock PM, Meltzer PS: A genome-based strategy uncovers frequent BRAF mutations in melanoma. Cancer Cell 2:5-7, 2002

18. Cohen Y, Xing M, Mambo E, et al: BRAF mutation in papillary thyroid carcinoma. J Natl Cancer Inst 95:625-627, 2003

19. Cohen MS, Hussain HB, Moley JF: Inhibition of medullary thyroid carcinoma cell proliferation and RET phosphorylation by tyrosine kinase inhibitors. Surgery 132:960-967, 2002

20. Singer G, Oldt R III, Cohen Y, et al: Mutations in BRAF and KRAS characterize the development of low-grade ovarian serous carcinoma. J Natl Cancer Inst 95:484-486, 2003

21. Tannapfel A, Sommerer F, Benicke M, et al: Mutations of the BRAF gene in cholangiocarcinoma but not in hepatocellular carcinoma. Gut 52:706-712, 2003

22. Lee JW, Soung YH, Park WS, et al: BRAF mutations in acute leukemias. Leukemia 18:170-172, 2004

23. Weber A, Langhanki L, Sommerer F, et al: Mutations of the BRAF gene in squamous cell carcinoma of the head and neck. Oncogene 22:4757-4759, 2003

24. Brose MS, Volpe P, Feldman M, et al: BRAF and RAS mutations in human lung cancer and melanoma. Cancer Res 62:6997-7000, 2002

25. Lee SH, Lee JW, Soung YH, et al: BRAF and KRAS mutations in stomach cancer. Oncogene 22:6942-6945, 2003

26. Gemignani ML, Schlaerth AC, Bogomolniy F, et al: Role of KRAS and BRAF gene mutations in mucinous ovarian carcinoma. Gynecol Oncol 90: 378-381, 2003

27. Lee JW, Yoo NJ, Soung YH, et al: BRAF mutations in non-Hodgkin's lymphoma. Br J Cancer 89:1958-1960, 2003

28. Oka H, Chatani Y, Hoshino R, et al: Constitutive activation of mitogen-activated protein (MAP) kinases in human renal cell carcinoma. Cancer Res 55:4182-4187, 1995

29. Carson EB, McMahon M, Baylin SB, et al: Ret gene silencing is associated with Raf-1-induced medullary thyroid carcinoma cell differentiation. Cancer Res 55:2048-2052, 1995

30. McFarlin DR, Gould MN: Rat mammary carcinogenesis induced by in situ expression of constitutive Raf kinase activity is prevented by tethering Raf to the plasma membrane. Carcinogenesis 24:1149-1153, 2003

31. Kerkhoff E, Fedorov LM, Siefken R, et al: Lung-targeted expression of the c-Raf-1 kinase in transgenic mice exposes a novel oncogenic character of the wild-type protein. Cell Growth Differ 11:185-190, 2000

32. Graziano SL, Pfeifer AM, Testa JR, et al: Involvement of the RAF1 locus, at band 3p25, in the 3p deletion of small-cell lung cancer. Genes Chromosomes Cancer 3:283-293, 1991

33. Patel BK, Ray S, Whiteside TL, et al: Correlation of constitutive activation of raf-1 with morphological transformation and abrogation of tyrosine phosphorylation of distinct sets of proteins in human squamous carcinoma cells. Mol Carcinog 18:1-6, 1997

34. Riva C, Lavieille JP, Reyt E, et al: Differential c-myc, c-jun, c-raf and p53 expression in squamous cell carcinoma of the head and neck: Implication in drug and radioresistance. Eur J Cancer B Oral Oncol 31B:384-391, 1995

35. Ikeda S, Sumii H, Akiyama K, et al: Amplification of both c-myc and c-raf-1 oncogenes in a human osteosarcoma. Jpn J Cancer Res 80: 6-9, 1989

36. Mitsunobu F, Fukui M, Oda T, et al: A mechanism of c-raf-1 activation: Fusion of the lipocortin II amino-terminal sequence with the c-raf-1 kinase domain. Oncogene 4:437-442, 1989

37. LaRocca RV, Rosenblum M, Westermark B, et al: Patterns of proto-oncogene expression in human glioma cell lines. J Neurosci Res 24: 97-106, 1989

38. Fukui M, Yamamoto T, Kawai S, et al: Molecular cloning and characterization of an activated human c-raf-1 gene. Mol Cell Biol 7:1776-1781, 1987

39. Korshunov A, Neben K, Wrobel G, et al: Gene expression patterns in ependymomas correlate with tumor location, grade, and patient age. Am J Pathol 163:1721-1727, 2003

40. Ting LP, Jeng KS, Chou CK, et al: Expression of oncogenes in human hepatoma cell lines. Zhonghua Min Guo Wei Sheng Wu Ji Mian Yi Xue Za Zhi 21:141-150, 1988

41. Jenke HS, Deml E, Oesterle D: C-raf expression in early rat liver tumorigenesis after promotion with polychlorinated biphenyls or phenobarbital. Xenobiotica 24:569-580, 1994

42. Beer DG, Neveu MJ, Paul DL, et al: Expression of the c-raf protooncogene, gamma-glutamyltranspeptidase, and gap junction protein in rat liver neoplasms. Cancer Res 48:1610-1617, 1988

43. Berger DH, Jardines LA, Chang H, et al: Activation of Raf-1 in human pancreatic adenocarcinoma. J Surg Res 69:199-204, 1997

44. Rowinsky EK, Windle JJ, Von Hoff DD: Ras protein farnesyltransferase: A strategic target for anticancer therapeutic development. J Clin Oncol 17:3631-3652, 1999

45. Yarden Y: The EGFR family and its ligands in human cancer: Signalling mechanisms and therapeutic opportunities. Eur J Cancer 37: S3-S8, 2001 (suppl 4)

46. Boguski MS, McCormick F: Proteins regulating Ras and its relatives. Nature 366:643-654, 1993

47. English JM, Cobb MH: Pharmacological inhibitors of MAPK pathways. Trends Pharmacol Sci 23:40-45, 2002

48. Kohno M, Pouyssegur J: Pharmacological inhibitors of the ERK signaling pathway: Application as anticancer drugs. Prog Cell Cycle Res 5:219-224, 2003

49. Liebmann C: Regulation of MAP kinase activity by peptide receptor signalling pathway: Paradigms of multiplicity. Cell Signal 13:777-785, 2001

50. Lewis TS, Shapiro PS, Ahn NG: Signal transduction through MAP kinase cascades. Adv Cancer Res 74:49-139, 1998

51. Kerkhoff E, Rapp UR: Cell cycle targets of Ras/Raf signalling. Oncogene 17:1457-1462, 1998

52. Chang F, Steelman LS, Shelton JG, et al: Regulation of cell cycle progression and apoptosis by the Ras/Raf/MEK/ERK pathway. Int J Oncol 22:469-480, 2003

53. Bos JL: Ras oncogenes in human cancer: A review. Cancer Res 49:4682-4689, 1989

54. Miyakis S, Sourvinos G, Spandidos DA: Differential expression and mutation of the ras family genes in human breast cancer. Biochem Biophys Res Commun 251:609-612, 1998

55. McCormick F: Ras biology in atomic detail. Nat Struct Biol 3:653-655, 1996

56. Hancock JF, Paterson H, Marshall CJ: A polybasic domain or palmitoylation is required in addition to the CAAX motif to localize p21ras to the plasma membrane. Cell 63:133-139, 1990

57. Kato K, Cox AD, Hisaka MM, et al: Isoprenoid addition to Ras protein is the critical modification for its membrane association and transforming activity. Proc Natl Acad Sci U S A 89:6403-6407, 1992

58. Polakis P, McCormick F: Interactions between p21ras proteins and their GTPase activating proteins. Cancer Surv 12:25-42, 1992

59. Clark GJ, Cox AD, Graham SM, et al: Biological assays for Ras transformation. Methods Enzymol 255:395-412, 1995

60. Clark GJ, Der CJ: Ras Proto-Oncogene Activation in Human Malignancy. Totowa, NJ, Humana Press, 1995

61. Barbacid M: Ras genes. Annu Rev Biochem 56:779-827, 1987

62. Gibbs JB, Oliff A: The potential of farnesyltransferase inhibitors as cancer chemotherapeutics. Annu Rev Pharmacol Toxicol 37: 143-166, 1997

63. Omer CA, Anthony NJ, Buser-Doepner CA, et al: Farnesyl: Proteintransferase inhibitors as agents to inhibit tumor growth. Biofactors 6:359-366, 1997

64. Whyte DB, Kirschmeier P, Hockenberry TN, et al: K- and N-Ras are geranylgeranylated in cells treated with farnesyl protein transferase inhibitors. J Biol Chem 272:14459-14464, 1997

65. Rowell CA, Kowalczyk JJ, Lewis MD, et al: Direct demonstration of geranylgeranylation and farnesylation of Ki-Ras in vivo. J Biol Chem 272:14093-14097, 1997

66. Prendergast GC, Oliff A: Farnesyltransferase inhibitors: Antineoplastic properties, mechanisms of action, and clinical prospects. Semin Cancer Biol 10:443-452, 2000

67. Marshall CJ: Ras effectors. Curr Opin Cell Biol 8:197-204, 1996

68. Khosravi-Far R, Campbell S, Rossman KL, et al: Increasing complexity of Ras signal transduction: Involvement of Rho family proteins. Adv Cancer Res 72:57-107, 1998

69. Smeal T, Binetruy B, Mercola DA, et al: Oncogenic and transcriptional cooperation with Ha-Ras requires phosphorylation of c-Jun on serines 63 and 73. Nature 354:494-496, 1991

70. Binetruy B, Smeal T, Karin M: Ha-Ras augments c-Jun activity and stimulates phosphorylation of its activation domain. Nature 351: 122-127, 1991

71. Pulverer BJ, Kyriakis JM, Avruch J, et al: Phosphorylation of c-jun mediated by MAP kinases. Nature 353:670-674, 1991

72. Derijard B, Raingeaud J, Barrett T, et al: Independent human MAP-kinase signal transduction pathways defined by MEK and MKK isoforms. Science 267:682-685, 1995

73. Bagrodia S, Derijard B, Davis RJ, et al: Cdc42 and PAK-mediated signaling leads to Jun kinase and p38 mitogen-activated protein kinase activation. J Biol Chem 270:27995-27998, 1995

74. Robinson MJ, Cobb MH: Mitogen-activated protein kinase pathways. Curr Opin Cell Biol 9:180-186, 1997

75. Dhanasekaran N, Premkumar Reddy E: Signaling by dual specificity kinases. Oncogene 17:1447-1455, 1998

76. Schaeffer HJ, Weber MJ: Mitogen-activated protein kinases: Specific messages from ubiquitous messengers. Mol Cell Biol 19: 2435-2444, 1999

77. Zhang XF, Settleman J, Kyriakis JM, et al: Normal and oncogenic p21ras proteins bind to the amino-terminal regulatory domain of c-Raf-1. Nature 364:308-313, 1993

78. Avruch J, Khokhlatchev A, Kyriakis JM, et al: Ras activation of the Raf kinase: Tyrosine kinase recruitment of the MAP kinase cascade. Recent Prog Horm Res 56:127-155, 2001

79. Marais R, Light Y, Mason C, et al: Requirement of Ras-GTP-Raf complexes for activation of Raf-1 by protein kinase C. Science 280: 109-112, 1998

80. Stokoe D, Macdonald SG, Cadwallader K, et al: Activation of Raf as a result of recruitment to the plasma membrane. Science 264:1463-1467, 1994

81. Pritchard C, McMahon M: Raf revealed in life-or-death decisions. Nat Genet 16:214-215, 1997

82. Morrison DK, Cutler RE: The complexity of Raf-1 regulation. Curr Opin Cell Biol 9:174-179, 1997

83. Marshall CJ: Cell signaling: Raf gets it together. Nature 383:127-128, 1996

84. Leevers SJ, Paterson HF, Marshall CJ: Requirement for Ras in Raf activation is overcome by targeting Raf to the plasma membrane. Nature 369:411-414, 1994

85. Mason CS, Springer CJ, Cooper RG, et al: Serine and tyrosine phosphorylations cooperate in Raf-1, but not B-Raf activation. Embo J 18: 2137-2148, 1999

86. Stancato LF, Sakatsume M, David M, et al: Beta interferon and oncostatin M activate Raf-1 and mitogen-activated protein kinase through a JAK1-dependent pathway. Mol Cell Biol 17:3833-3840, 1997

87. Farrar MA, Alberol I, Perlmutter RM: Activation of the Raf-1 kinase cascade by coumermycin-induced dimerization. Nature 383: 178-181, 1996

88. Kolch W, Heidecker G, Kochs G, et al: Protein kinase C alpha activates RAF-1 by direct phosphorylation. Nature 364:249-252, 1993

89. Yao B, Zhang Y, Delikat S, et al: Phosphorylation of Raf by ceramide-activated protein kinase. Nature 378:307-310, 1995

90. Kasid U, Suy S, Dent P, et al: Activation of Raf by ionizing radiation. Nature 382:813-816, 1996

91. Hoyos B, Imam A, Korichneva I, et al: Activation of c-Raf kinase by ultraviolet light: Regulation by retinoids. J Biol Chem 277:23949-23957, 2002

92. Tilbrook PA, Colley SM, McCarthy DJ, et al: Erythropoietin-stimulated Raf-1 tyrosine phosphorylation is associated with the tyrosine kinase Lyn in J2E erythroleukemic cells. Arch Biochem Biophys 396:128-132, 2001

93. Luo Z, Tzivion G, Belshaw PJ, et al: Oligomerization activates c-Raf-1 through a Ras-dependent mechanism. Nature 383:181-185, 1996

94. Weber CK, Slupsky JR, Kalmes HA, et al: Active Ras induces heterodimerization of cRaf and BRaf. Cancer Res 61:3595-3598, 2001

95. Hekman M, Hamm H, Villar AV, et al: Associations of B- and C-Raf with cholesterol, phosphatidylserine, and lipid second messengers: Preferential binding of Raf to artificial lipid rafts. J Biol Chem 277:24090-24102, 2002

96. Cook SJ, McCormick F: Inhibition by cAMP of Ras-dependent activation of Raf. Science 262:1069-1072, 1993

97. Hafner S, Adler HS, Mischak H, et al: Mechanism of inhibition of Raf-1 by protein kinase A. Mol Cell Biol 14:6696-6703, 1994

98. Reusch HP, Zimmermann S, Schaefer M, et al: Regulation of Raf by Akt controls growth and differentiation in vascular smooth muscle cells. J Biol Chem 276:33630-33637, 2001

99. Jansen HW, Lurz R, Bister K, et al: Homologous cell-derived oncogenes in avian carcinoma virus MH2 and murine sarcoma virus 3611. Nature 307:281-284, 1984

100. Sutrave P, Bonner TI, Rapp UR, et al: Nucleotide sequence of avian retroviral oncogene v-mil: Homologue of murine retroviral oncogene v-raf. Nature 309:85-88, 1984

101. Kan NC, Flordellis CS, Mark GE, et al: A common onc gene sequence transduced by avian carcinoma virus MH2 and by murine sarcoma virus 3611. Science 223:813-816, 1984

102. Rapp UR, Goldsborough MD, Mark GE, et al: Structure and biological activity of v-raf, a unique oncogene transduced by a retrovirus. Proc Natl Acad Sci U S A 80:4218-4222, 1983

103. Calogeraki I, Barnier JV, Eychene A, et al: Genomic organization and nucleotide sequence of the coding region of the chicken c-Rmil(B-raf-1) proto-oncogene. Biochem Biophys Res Commun 193:1324-1331, 1993

104. Eychene A, Barnier JV, Apiou F, et al: Chromosomal assignment of two human B-raf(Rmil) proto-oncogene loci: B-raf-1 encoding the p94Braf/Rmil and B-raf-2, a processed pseudogene. Oncogene 7:1657-1660, 1992

105. Jaiswal RK, Weissinger E, Kolch W, et al: Nerve growth factor-mediated activation of the mitogen-activated protein (MAP) kinase cascade involves a signaling complex containing B-Raf and HSP90. J Biol Chem 271:23626-23629, 1996

106. Barnier JV, Papin C, Eychene A, et al: The mouse B-raf gene encodes multiple protein isoforms with tissue-specific expression. J Biol Chem 270:23381-23389, 1995

107. Papin C, Barnier JV, Eychene A, et al: B-raf gene encodes for multiple isoforms with Mek-1 kinase activity. C R Seances Soc Biol Fil 189:71-85, 1995

108. Nantel A, Huber M, Thomas DY: Localization of endogenous Grb10 to the mitochondria and its interaction with the mitochondrial-associated Raf-1 pool. J Biol Chem 274:35719-35724, 1999

109. Majewski M, Nieborowska-Skorska M, Salomoni P, et al: Activation of mitochondrial Raf-1 is involved in the antiapoptotic effects of Akt. Cancer Res 59:2815-2819, 1999

110. Wang HG, Rapp UR, Reed JC: Bcl-2 targets the protein kinase Raf-1 to mitochondria. Cell 87:629-638, 1996

111. Yuryev A, Ono M, Goff SA, et al: Isoform-specific localization of A-RAF in mitochondria. Mol Cell Biol 20:4870-4878, 2000

112. Morice C, Nothias F, Konig S, et al: Raf-1 and B-Raf proteins have similar regional distributions but differential subcellular localization in adult rat brain. Eur J Neurosci 11:1995-2006, 1999

113. Hagemann C, Rapp UR: Isotype-specific functions of Raf kinases. Exp Cell Res 253:34-46, 1999

114. Pritchard CA, Bolin L, Slattery R, et al: Post-natal lethality and neurological and gastrointestinal defects in mice with targeted disruption of the A-Raf protein kinase gene. Curr Biol 6:614-617, 1996

115. Houslay MD, Kolch W: Cell-type specific integration of cross-talk between extracellular signal-regulated kinase and cAMP signaling. Mol Pharmacol 58:659-668, 2000

116. Vojtek AB, Hollenberg SM, Cooper JA: Mammalian Ras interacts directly with the serine/threonine kinase Raf. Cell 74:205-214, 1993

117. Drugan JK, Khosravi-Far R, White MA, et al: Ras interaction with two distinct binding domains in Raf-1 may be required for Ras transformation. J Biol Chem 271:233-237, 1996

118. Brtva TR, Drugan JK, Ghosh S, et al: Two distinct Raf domains mediate interaction with Ras. J Biol Chem 270:9809-9812, 1995

119. Guan KL, Figueroa C, Brtva TR, et al: Negative regulation of the serine/threonine kinase B-Raf by Akt. J Biol Chem 275:27354-27359, 2000

120. Morrison DK, Heidecker G, Rapp UR, et al: Identification of the major phosphorylation sites of the Raf-1 kinase. J Biol Chem 268: 17309-17316, 1993

121. Zimmermann S, Moelling K: Phosphorylation and regulation of Raf by Akt (protein kinase B). Science 286:1741-1744, 1999

122. Mikula M, Schreiber M, Husak Z, et al: Embryonic lethality and fetal liver apoptosis in mice lacking the c-raf-1 gene. Embo J 20:1952-1962, 2001

123. Wu X, Noh SJ, Zhou G, et al: Selective activation of MEK1 but not MEK2 by A-Raf from epidermal growth factor-stimulated Hela cells. J Biol Chem 271:3265-3271, 1996

124. Marais R, Light Y, Paterson HF, et al: Differential regulation of Raf-1, A-Raf, and B-Raf by oncogenic ras and tyrosine kinases. J Biol Chem 272:4378-4383, 1997

125. Pritchard CA, Samuels ML, Bosch E, et al: Conditionally oncogenic forms of the A-Raf and B-Raf protein kinases display different biological and biochemical properties in NIH 3T3 cells. Mol Cell Biol 15:6430-6442, 1995

126. Kubicek M, Pacher M, Abraham D, et al: Dephosphorylation of Ser-259 regulates Raf-1 membrane association. J Biol Chem 277:7913-7919, 2002

127. Chuang E, Barnard D, Hettich L, et al: Critical binding and regulatory interactions between Ras and Raf occur through a small, stable N-terminal domain of Raf and specific Ras effector residues. Mol Cell Biol 14:5318-5325, 1994

128. Voice JK, Klemke RL, Le A, et al: Four human ras homologs differ in their abilities to activate Raf-1, induce transformation, and stimulate cell motility. J Biol Chem 274:17164-17170, 1999

129. Okada T, Hu CD, Jin TG, et al: The strength of interaction at the Raf cysteine-rich domain is a critical determinant of response of Raf to Ras family small GTPases. Mol Cell Biol 19:6057-6064, 1999

130. Yamamori B, Kuroda S, Shimizu K, et al: Purification of a Ras-dependent mitogen-activated protein kinase kinase kinase from bovine brain cytosol and its identification as a complex of B-Raf and 14-3-3 proteins. J Biol Chem 270:11723-11726, 1995

131. Vossler MR, Yao H, York RD, et al: CAMP activates MAP kinase and Elk-1 through a B-Raf- and Rap1-dependent pathway. Cell 89:73-82, 1997

132. York RD, Yao H, Dillon T, et al: Rap1 mediates sustained MAP kinase activation induced by nerve growth factor. Nature 392:622-626, 1998

133. Bos JL, de Rooij J, Reedquist KA: Rap1 signalling: Adhering to new models. Nat Rev Mol Cell Biol 2:369-377, 2001

134. Chaudhary A, King WG, Mattaliano MD, et al: Phosphatidylinositol 3-kinase regulates Raf1 through Pak phosphorylation of serine 338. Curr Biol 10:551-554, 2000

135. Diaz B, Barnard D, Filson A, et al: Phosphorylation of Raf-1 serine 338-serine 339 is an essential regulatory event for Ras-dependent activation and biological signaling. Mol Cell Biol 17:4509-4516, 1997

136. King AJ, Sun H, Diaz B, et al: The protein kinase Pak3 positively regulates Raf-1 activity through phosphorylation of serine 338. Nature 396:180-183, 1998

137. Dhillon AS, Meikle S, Yazici Z, et al: Regulation of Raf-1 activation and signalling by dephosphorylation. Embo J 21:64-71, 2002

138. Zhang BH, Tang ED, Zhu T, et al: Serum- and glucocorticoid-inducible kinase SGK phosphorylates and negatively regulates B-Raf. J Biol Chem 276:31620-31626, 2001

139. Dhillon AS, Meikle S, Peyssonnaux C, et al: A Raf-1 mutant that dissociates MEK/extracellular signal-regulated kinase activation from malignant transformation and differentiation but not proliferation. Mol Cell Biol 23:1983-1993, 2003

140. Chong H, Lee J, Guan KL: Positive and negative regulation of Raf kinase activity and function by phosphorylation. Embo J 20:3716-3727, 2001

141. Fabian JR, Daar IO, Morrison DK: Critical tyrosine residues regulate the enzymatic and biological activity of Raf-1 kinase. Mol Cell Biol 13:7170-7179, 1993

142. Sun H, King AJ, Diaz HB, et al: Regulation of the protein kinase Raf-1 by oncogenic Ras through phosphatidylinositol 3-kinase, Cdc42/Rac and Pak. Curr Biol 10:281-284, 2000

143. Campbell S, Otis M, Cote M, et al: Connection between integrins and cell activation in rat adrenal glomerulosa cells: A role for Arg-Gly-Asp peptide in the activation of the p42/p44(mapk) pathway and intracellular calcium. Endocrinology 144:1486-1495, 2003

144. Zhang BH, Guan KL: Activation of B-Raf kinase requires phosphorylation of the conserved residues Thr598 and Ser601. Embo J 19:5429-5439, 2000

145. Wojnowski L, Stancato LF, Larner AC, et al: Overlapping and specific functions of Braf and Craf-1 proto-oncogenes during mouse embryogenesis. Mech Dev 91:97-104, 2000

146. Dent P, Haser W, Haystead TA, et al: Activation of mitogen-activated protein kinase kinase by v-Raf in NIH 3T3 cells and in vitro. Science 257:1404-1407, 1992

147. Catling AD, Reuter CW, Cox ME, et al: Partial purification of a mitogen-activated protein kinase kinase activator from bovine brain: Identification as B-Raf or a B-Raf-associated activity. J Biol Chem 269:30014-30021, 1994

148. Catling AD, Schaeffer HJ, Reuter CW, et al: A proline-rich sequence unique to MEK1 and MEK2 is required for raf binding and regulates MEK function. Mol Cell Biol 15:5214-5225, 1995

149. Howe LR, Leevers SJ, Gomez N, et al: Activation of the MAP kinase pathway by the protein kinase raf. Cell 71:335-342, 1992

150. Kyriakis JM, App H, Zhang XF, et al: Raf-1 activates MAP kinase-kinase. Nature 358:417-421, 1992

151. Jaiswal RK, Moodie SA, Wolfman A, et al: The mitogen-activated protein kinase cascade is activated by B-Raf in response to nerve growth factor through interaction with p21ras. Mol Cell Biol 14:6944-6953, 1994

152. Yan M, Templeton DJ: Identification of 2 serine residues of MEK-1 that are differentially phosphorylated during activation by raf and MEK kinase. J Biol Chem 269:19067-19073, 1994

153. Reuter CW, Catling AD, Jelinek T, et al: Biochemical analysis of MEK activation in NIH3T3 fibroblasts: Identification of B-Raf and other activators. J Biol Chem 270:7644-7655, 1995

154. Kolch W: Meaningful relationships: The regulation of the Ras/Raf/MEK/ERK pathway by protein interactions. Biochem J 351:289-305, 2000

155. Baumann B, Weber CK, Troppmair J, et al: Raf induces NF-kappaB by membrane shuttle kinase MEKK1, a signaling pathway critical for transformation. Proc Natl Acad Sci U S A 97:4615-4620, 2000

156. Wang S, Ghosh RN, Chellappan SP: Raf-1 physically interacts with Rb and regulates its function: A link between mitogenic signaling and cell cycle regulation. Mol Cell Biol 18:7487-7498, 1998

157. Wang HG, Miyashita T, Takayama S, et al: Apoptosis regulation by interaction of Bcl-2 protein and Raf-1 kinase. Oncogene 9:2751-2756, 1994

158. Papin C, Denouel A, Calothy G, et al: Identification of signalling proteins interacting with B-Raf in the yeast two-hybrid system. Oncogene 12:2213-2221, 1996

159. Ahn NG, Seger R, Bratlien RL, et al: Multiple components in an epidermal growth factor-stimulated protein kinase cascade: In vitro activation of a myelin basic protein/microtubule-associated protein 2 kinase. J Biol Chem 266:4220-4227, 1991

160. Crews CM, Alessandrini A, Erikson RL: The primary structure of MEK, a protein kinase that phosphorylates the ERK gene product. Science 258:478-480, 1992

161. Morrison DK: KSR: A MAPK scaffold of the Ras pathway? J Cell Sci 114:1609-1612, 2001

162. Michaud NR, Therrien M, Cacace A, et al: KSR stimulates Raf-1 activity in a kinase-independent manner. Proc Natl Acad Sci U S A 94:12792-12796, 1997

163. Galaktionov K, Jessus C, Beach D: Raf1 interaction with Cdc25 phosphatase ties mitogenic signal transduction to cell cycle activation. Genes Dev 9:1046-1058, 1995

164. Jinno S, Suto K, Nagata A, et al: Cdc25A is a novel phosphatase functioning early in the cell cycle. Embo J 13:1549-1556, 1994

165. Janosch P, Kieser A, Eulitz M, et al: The Raf-1 kinase associates with vimentin kinases and regulates the structure of vimentin filaments. FASEB J 14:2008-2021, 2000

166. Li S, Sedivy JM: Raf-1 protein kinase activates the NF-kappa B transcription factor by dissociating the cytoplasmic NF-kappa B-I kappa B complex. Proc Natl Acad Sci U S A 90:9247-9251, 1993

167. Peruzzi F, Prisco M, Dews M, et al: Multiple signaling pathways of the insulin-like growth factor 1 receptor in protection from apoptosis. Mol Cell Biol 19:7203-7215, 1999

168. Patriotis C, Russeva MG, Lin JH, et al: Tpl-2 induces apoptosis by promoting the assembly of protein complexes that contain caspase-9, the adapter protein Tvl-1, and procaspase-3. J Cell Physiol 187:176-187, 2001

169. Chen J, Fujii K, Zhang L, et al: Raf-1 promotes cell survival by antagonizing apoptosis signal-regulating kinase 1 through a MEK-ERK independent mechanism. Proc Natl Acad Sci U S A 98:7783-7788, 2001

170. Jamal S, Ziff EB: Raf phosphorylates p53 in vitro and potentiates p53-dependent transcriptional transactivation in vivo. Oncogene 10:2095-2101, 1995

171. Nilsson I, Hoffmann I: Cell cycle regulation by the Cdc25 phosphatase family. Prog Cell Cycle Res 4:107-114, 2000

172. Cornwell MM, Smith DE: A signal transduction pathway for activation of the mdr1 promoter involves the proto-oncogene c-raf kinase. J Biol Chem 268:15347-15350, 1993

173. Harris P, Morton CC, Guglielmi P, et al: Mapping by chromosome sorting of several gene probes, including c-myc, to the derivative

chromosomes of a 3;8 translocation associated with familial renal cancer. Cytometry 7:589-594, 1986

174. Miyamoto K, Tomita N, Ishii A, et al: Specific abnormalities of chromosome 14 in patients with acute type of adult T-cell leukemia/lymphoma. Int J Cancer 40:461-468, 1987

175. Nagao M, Ishikawa F, Tahira T, et al: Activation of rat and human c-raf(-1) by rearrangement. Princess Takamatsu Symp 17:75-84, 1986

176. Huser M, Luckett J, Chiloeches A, et al: MEK kinase activity is not necessary for Raf-1 function. Embo J 20:1940-1951, 2001

177. Niehof M, Radziwill G, Klauser S, et al: A small peptide derived from the aminoterminus of c-Raf-1 inhibits c-Raf-1/Ras binding. Biochem Biophys Res Commun 206:46-50, 1995

178. Metzelaar-Blok JA, Jager MJ, Moghaddam PH, et al: Frequent loss of heterozygosity on chromosome 6p in uveal melanoma. Hum Immunol 60:962-969, 1999

179. Cohen Y, Goldenberg-Cohen N, Parrella P, et al: Lack of BRAF mutation in primary uveal melanoma. Invest Ophthalmol Vis Sci 44:2876-2878, 2003

180. Pollock PM, Harper UL, Hansen KS, et al: High frequency of BRAF mutations in nevi. Nat Genet 33:19-20, 2003

181. Takayama T, Ohi M, Hayashi T, et al: Analysis of K-ras, APC, and beta-catenin in aberrant crypt foci in sporadic adenoma, cancer, and familial adenomatous polyposis. Gastroenterology 121:599-611, 2001

182. Yamashita N, Minamoto T, Ochiai A, et al: Frequent and characteristic K-ras activation in aberrant crypt foci of colon: Is there preference among K-ras mutants for malignant progression? Cancer 75:1527-1533, 1995

183. Busca R, Abbe P, Mantoux F, et al: Ras mediates the cAMP-dependent activation of extracellular signal-regulated kinases (ERKs) in melanocytes. Embo J 19:2900-2910, 2000

184. Iacovelli L, Capobianco L, Salvatore L, et al: Thyrotropin activates mitogen-activated protein kinase pathway in FRTL-5 by a cAMP-dependent protein kinase A-independent mechanism. Mol Pharmacol 60:924-933, 2001

185. Manning G, Whyte DB, Martinez R, et al: The protein kinase complement of the human genome. Science 298:1912-1934, 2002

186. Stanton VP Jr, Cooper GM: Activation of human raf transforming genes by deletion of normal amino-terminal coding sequences. Mol Cell Biol 7:1171-1179, 1987

187. Kasid U, Pfeifer A, Weichselbaum RR, et al: The raf oncogene is associated with a radiation-resistant human laryngeal cancer. Science 237:1039-1041, 1987

188. Yu X, Guo ZS, Marcu MG, et al: Modulation of p53, ErbB1, ErbB2, and Raf-1 expression in lung cancer cells by depsipeptide FR901228. J Natl Cancer Inst 94:504-513, 2002

189. Carter G, Lemoine NR: Antisense technology for cancer therapy: Does it make sense? Br J Cancer 67:869-876, 1993

190. Stephens AC, Rivers RP: Antisense oligonucleotide therapy in cancer. Curr Opin Mol Ther 5:118-122, 2003

191. Monia BP, Sasmor H, Johnston JF, et al: Sequence-specific antitumor activity of a phosphorothioate oligodeoxyribonucleotide targeted to human C-raf kinase supports an antisense mechanism of action in vivo. Proc Natl Acad Sci U S A 93:15481-15484, 1996

192. Monia BP, Johnston JF, Geiger T, et al: Antitumor activity of a phosphorothioate antisense oligodeoxynucleotide targeted against C-raf kinase. Nat Med 2:668-675, 1996

193. Gokhale PC, McRae D, Monia BP, et al: Antisense raf oligodeoxyribonucleotide is a radiosensitizer in vivo. Antisense Nucleic Acid Drug Dev 9:191-201, 1999

194. Henry SP, Monteith D, Bennett F, et al: Toxicological and pharmacokinetic properties of chemically modified antisense oligonucleotide inhibitors of PKC-alpha and C-raf kinase. Anticancer Drug Des 12:409-420, 1997

195. Phillips JA, Craig SJ, Bayley D, et al: Pharmacokinetics, metabolism, and elimination of a 20-mer phosphorothioate oligodeoxynucleotide (CGP 69846A) after intravenous and subcutaneous administration. Biochem Pharmacol 54:657-668, 1997

196. Geary RS, Leeds JM, Fitchett J, et al: Pharmacokinetics and metabolism in mice of a phosphorothioate oligonucleotide antisense inhibitor of C-raf-1 kinase expression. Drug Metab Dispos 25:1272-1281, 1997

197. Cunningham CC, Holmlund JT, Schiller JH, et al: A phase I trial of c-Raf kinase antisense oligonucleotide ISIS 5132 administered as a continuous intravenous infusion in patients with advanced cancer. Clin Cancer Res 6:1626-1631, 2000

198. Stevenson JP, Yao KS, Gallagher M, et al: Phase I clinical/pharmacokinetic and pharmacodynamic trial of the c-raf-1 antisense oligonucleotide ISIS 5132 (CGP 69846A). J Clin Oncol 17:2227-2236, 1999

199. Rudin CM, Holmlund J, Fleming GF, et al: Phase I trial of ISIS 5132, an antisense oligonucleotide inhibitor of c-raf-1, administered by 24-hour weekly infusion to patients with advanced cancer. Clin Cancer Res 7:1214-1220, 2001

200. Tolcher AW, Reyno L, Venner PM, et al: A randomized phase II and pharmacokinetic study of the antisense oligonucleotides ISIS 3521 and ISIS 5132 in patients with hormone-refractory prostate cancer. Clin Cancer Res 8:2530-2535, 2002

201. Cripps MC, Figueredo AT, Oza AM, et al: Phase II randomized study of ISIS 3521 and ISIS 5132 in patients with locally advanced or metastatic colorectal cancer: A National Cancer Institute of Canada Clinical Trials Group study. Clin Cancer Res 8:2188-2192, 2002

202. Oza AM, Elit L, Swenerton K, et al: Phase II study of CGP 69846A (ISIS 5132) in recurrent epithelial ovarian cancer: An NCIC Clinical Trials Group study (NCIC IND. 116). Gynecol Oncol 89:129-133, 2003

203. Coudert B, Anthoney A, Fiedler W, et al: Phase II trial with ISIS 5132 in patients with small-cell (SCLC) and non-small cell (NSCLC) lung cancer: A European Organization for Research and Treatment of Cancer (EORTC) Early Clinical Studies Group report. Eur J Cancer 37: 2194-2198, 2001

204. Bonner TI, Kerby SB, Sutrave P, et al: Structure and biological activity of human homologs of the raf/mil oncogene. Mol Cell Biol 5:1400-1407, 1985

205. Wan PT, Garnett MJ, Roe SM, et al: Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. Cell 116:855-867, 2004

206. Wilhelm S, Chien DS: BAY 43-9006: Preclinical data. Curr Pharm Des 8:2255-2257, 2002

207. Choi YJ, Fanidi A, White S, et al: The novel inhibitor BAY 43-9006 blocks signaling and proliferation in b-Raf mutant and wild type melanoma and colorectal tumor cell lines. Presented at Am Assoc Cancer Res 94th Annual Meeting, Washington, DC, July11-14, 2003

208. Wilhelm S, Carter C, Tang L, et al: BAY 43-9006 exhibits broad spectrum anti-tumor activity and targets raf/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. Presented at the 2003 AACR-NCI-EORTC International Conference for Molecular Targets and Cancer Therapeutics, Boston, MA, November 17-21, 2003

209. Carter C, Deuringer P, et al: Investigators Brochure BAY 43-9006/ Raf Kinase Inhibitor Version No. 4.1. West Haven, CT, Bayer Pharmaceuticals Corporation, 2003

210. Wilhelm SM, Carter C, Tang LY, et al: BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. Cancer Res 64:7099-7109, 2004

211. Alavi A, Hood JD, Frausto R, et al: Role of Raf in vascular protection from distinct apoptotic stimuli. Science 301:94-96, 2003

212. Vincent P, Zhang X, Chen CM, et al: Preclinical chemotherapy with the Raf kinase inhibitor BAY 43-9006 in combination with gefitinib, vinorelbine, gemcitabine and doxorubicin. Presented at Am Assoc Cancer Res 94th Annual Meeting, Washington, DC, July11-14, 2003

213. Lyons JF, Wilhelm S, Hibner B, et al: Discovery of a novel Raf kinase inhibitor. Endocr Relat Cancer 8:219-225, 2001

214. Activity of the Raf kinase inhibitor BAY 43-9006 in patients with advanced solid tumors. Clin Colorectal Cancer 3:16-18, 2003

215. Strumberg D, Voliotis D, Moeller JG, et al: Results of phase I pharmacokinetic and pharmacodynamic studies of the Raf kinase inhibitor BAY 43-9006 in patients with solid tumors. Int J Clin Pharmacol Ther 40:580-581, 2002

216. Hilger RA, Kredke S, Hedley D, et al: Inhibition of ERK phosphorylation in patients treated with the Raf kinase inhibitor BAY 43-9006. Eur J Cancer 38:S52-S53, 2002

217. Kopec JA, Abrahamowicz M, Esdaile JM: Randomized discontinuation trials: Utility and efficiency. J Clin Epidemiol 46:959-971, 1993

218. Ratain MJ, Flaherty K, Stadler WM, et al: Preliminary antitumor activity of BAY 43-9006 in metastatic renal cell carcinoma and other advanced refractory solid tumors in a phase II randomized discontinuation trial (RDT). J Clin Oncol 22:381s, 2004 (suppl; abstr 4501)

219. Ratain MJ, Eisen T, Stadler WM: Final findings from a Phase II, placebo-controlled, randomized discontinuation trial (RDT) of sorafenib (BAY 43-9006) in patients with advanced renal cell carcinoma (RCC). J Clin Oncol 23:388s, 2005 (suppl; abstr 4544)

220. Escudier B, Szczylik C, Eisen T, et al: Randomized phase III trial of the Raf kinase and VEGFR inhibitor sorafenib (BAY 43-9006) in patients with advanced renal cell carcinoma (RCC). Proc Am Soc Clin Oncol 23:380s, 2005 (suppl; abstr LBA4510)

221. Ahmad T, Marias R, Pyle L, et al: BAY 43-9006 in patients with advanced melanoma: The Royal Marsden experience. J Clin Oncol 22:708s, 2004 (suppl; abstr 7506)

222. Flaherty KT, Brose M, Schuchter L, et al: Phase I/II trial of BAY 43-9006, carboplatin (C) and paclitaxel (P) demonstrates preliminary antitumor activity in the expansion cohort of patients with metastatic melanoma. J Clin Oncol 22: 708s, 2004 (suppl; abstr 7507)

223. Siu LL, Takimoto CH, Awada A, et al: A phase I/II trial of BAY 43-9006 and gemcitabine in advanced solid tumors and in advanced pancreatic cancer. J Clin Oncol 22:209s, 2004 (suppl; abstr 3059)

224. Richly H, Kupsch P, Passage K, et al: A phase I clinical and pharmacokinetic study of the Raf kinase inhibitor (RKI) BAY 43-9006 administered in combination with doxorubicin in patients with solid tumors. Int J Clin Pharmacol Ther 41:620-621, 2003

225. Heim M, Sharifi M, Hilger RA, et al: Antitumor effect and potentiation or reduction in cytotoxic drug activity in human colon carcinoma cells by the Raf kinase inhibitor (RKI) BAY 43-9006. Int J Clin Pharmacol Ther 41:616-617, 2003

226. Mross K, Steinbild S, Baas F, et al: Drug-drug interaction pharmacokinetic study with the Raf kinase inhibitor (RKI) BAY 43-9006 administered in combination with irinotecan (CPT-11) in patients with solid tumors. Int J Clin Pharmacol Ther 41:618-619, 2003

227. Kupsch P, Passarge K, Richly H, et al: Results of a phase I trial of BAY 43-9006 in combination with oxaliplatin in patients with refractory solid tumors. J Clin Oncol 22:209s, 2004 (suppl; abstr 3056)

228. Heimbrook HE, Huber SM, Stirdivant D, et al: Identification of potent selective kinase inhibitors of Raf. Presented at Am Assoc Cancer Res 89th Annual Meeting, New Orleans, LA, March 28-April 1, 1998 (abstr 3793)

229. Shelton JG, Moye PW, Steelman LS, et al: Differential effects of kinase cascade inhibitors on neoplastic and cytokine-mediated cell proliferation. Leukemia 17:1765-1782, 2003

230. Lackey K, Cory M, Davis R, et al: The discovery of potent cRaf1 kinase inhibitors. Bioorg Med Chem Lett 10:223-226, 2000

231. Wood E, Crosby RM, Dickerson S, et al: A prodrug approach to the design of cRaf1 kinase inhibitors with improved cellular activity. Anticancer Drug Des 16:1-6, 2001

232. Hall-Jackson CA, Goedert M, Hedge P, et al: Effect of SB 203580 on the activity of c-Raf in vitro and in vivo. Oncogene 18:2047-2054, 1999

233. Maloney A, Workman P: HSP90 as a new therapeutic target for cancer therapy: The story unfolds. Expert Opin Biol Ther 2:3-24, 2002

234. Sausville EA, Tomaszewski JE, Ivy P: Clinical development of 17-allylamino, 17-demethoxy-geldanamycin. Curr Cancer Drug Targets 3:377-383, 2003

235. Ikuina Y, Amishiro N, Miyata M, et al: Synthesis and antitumor activity of novel O-carbamoylmethyloxime derivatives of radicicol. J Med Chem 46:2534-2541, 2003

236. Hood JD, Bednarski M, Frausto R, et al: Tumor regression by targeted gene delivery to the neovasculature. Science 296:2404-2407, 2002

DOCUMENT-IDENTIFIER: US 6103692 A
** See image for Certificate of Correction **
TITLE: Inhibiting protein interactions

Detailed Description Text (63):
Candidate compounds can be evaluated for anti-proliferative activity by contacting Raf or a Ras-binding fragment thereof, e.g., a zinc finger domain-containing fragment of Raf, with a candidate compound and determining binding of the candidate compound to the peptide, or Ras-Raf binding. Raf or Ras-binding fragment of Raf can be immobilized using methods known in the art such as binding a GST-Raf fusion protein to a polymeric bead containing glutathione. Binding of the compound to the Raf peptide is correlated with the ability of the compound to disrupt the signal transduction pathway and thus inhibit cell proliferation.

Detailed Description Text (66):
In another screening method, one of the components of the Ras-Raf binding complex, such as Ras or a Raf-binding fragment of Ras or Raf or a Ras-binding fragment of Raf, is immobilized. Peptides can be immobilized using methods known in the art, such as adsorption onto a plastic microtiter plate or specific binding of a GST-fusion protein to a polymeric bead containing glutathione. For example, GST-Raf or GST-Ras can be bound to glutathione-Sepharose beads. The immobilized peptide is then contacted with the labeled peptide to which it binds (Ras in this case) in the presence and absence of a candidate compound. Unbound peptide can then be removed and the complex solubilized and analyzed to determine the amount of bound labeled peptide. A decrease in binding is an indication that the candidate compound inhibits the interaction of Ras with Raf.

Detailed Description Text (67):
A variation of the above-described screening method can be used to screen for another class of candidate compounds which are capable of disrupting a previously-formed Ras-Raf interaction. In this example, a complex comprising Ras or a Raf-binding fragment thereof bound to Raf or a Ras-binding fragment thereof is immobilized as described above and contacted with a candidate compound. The dissolution of the complex by the candidate compound correlates with the ability of the candidate compound to disrupt or inhibit the interaction of Ras with Raf.

Previous Doc Next Doc Go to Doc#

US006103692A

United States Patent [19]

Avruch et al.

[11] Patent Number: 6,103,692

[45] Date of Patent: Aug. 15, 2000

[54] **INHIBITING PROTEIN INTERACTIONS**

[75] Inventors: **Joseph Avruch**, Brookline; **Zhujun Luo**, Chestnut Hill, both of Mass.; **Mark S. Marshall**, Carmel, Ind.

[73] Assignee: **The General Hospital Corporation**, Boston, Mass.

[21] Appl. No.: **08/814,836**

[22] Filed: **Mar. 11, 1997**

Related U.S. Application Data

[60] Provisional application No. 60/013,274, Mar. 12, 1996.

[51] **Int. Cl.**[7] **A61K 38/00**

[52] **U.S. Cl.** **514/12**; 514/13; 514/14; 514/15

[58] **Field of Search** 514/12–15

[56] **References Cited**

U.S. PATENT DOCUMENTS

5,582,995 12/1996 Avruch et al. 435/71
5,597,719 1/1997 Freed et al. 435/194

FOREIGN PATENT DOCUMENTS

29727 12/1994 WIPO .

OTHER PUBLICATIONS

Andersson et al., "Cloning, Structure, and Expression of the Mitochondrial Cytochrome P–450 Sterol 26–Hydro–xylase, a Bile Acid Biosynthetic Enzyme", *J. Biol. Chem.*, 264:8222–29 (1989).

Avruch et al., "Raf meets Ras: Completing the Framework of a Signal Transduction Pathway", *TIBS*, 19:279–83 (1994).

Barnard et al., "Identification of the Sites of Interaction Between c–Raf–1 and Ras–GTP", *Oncogene*, 10:1283–90 (1995).

Bodor et al., "A Strategy for Delivering Peptides into the Central Nervous System by Sequential Metabolism", *Science*, 257:1698–1700 (1992).

Brtva et al., "Two Distinct Raf Domains Mediate Interaction with Ras", *J. Biol. Chem.*, 270:9809–12 (1995).

Cadwallader et al., "N–Terminally Myristoylated Ras Proteins Require Palmitoylation or a Polybasic Domain for Plasma Membrane Localization", *Mol. and Cell. Biol.*, 14(7):4722–30 (1994).

Chow et al., "Functional Mapping of the N–terminal Regulatory Domain in the Human Raf–1 Protein Kinase", *J. Biol. Chem.*, 270:14100–106 (1995).

Chuang et al., "Critical Binding and Regulatory Interactions Between Ras and Raf Occur Through a Small, Stable N–terminal Domain of Raf and Specific Ras Effector Residues", *Mol. Cell. Biol.*, 14:5318–25 (1994).

Friden et al., "Blood–Brain Barrier Penetration and in Vivo Activity of an NGF Conjugate", *Science*, 259:343–77 (1993).

Fridman et al., "The Minimal Fragments of c–Raf–1 and NF1 That Can Suppress v–Ha–Ras–induced Malignant Phenotype", *J. Biol. Chem.*, 269:30105–108 (1994).

Ghosh et al., "Identification of Discrete Segments of Human Raf–1 Kinase Critical for High Affinity Binding to Ha–Ras", *J. Biol. Chem.*, 269:30785–88 (1994).

Ghosh et al., "The Cysteine–rich Region of Raf–1 Kinase Contains Zinc, Translocates to Liposomes, and is Adjacent to a Segment that Binds GTP–Ras", *J. Biol. Chem.*, 269:10000–7 (1994).

Guan et al., "Eukaryotic Proteins Expressin in *Escherichia coli*: An Improved Thrombin Cleavage and Purification Procedure of Fusion Proteins with Glutathione S–Transferase", *Anal. Biochem.*, 192:262–76 (1991).

Häfner et al., "Mechanism of Inhibition of Raf–1 by Protein Kinase A", *Mol. and Cell Biol.*, 14:6696–703 (1994).

Herrmann et al., "Quantitative Analysis of the Complex Between $p21^{ras}$ and the Ras–binding Domain of the Human Raf–1 Protein Kinase", *J. Biol. Chem.*, 270:2901–905 (1995).

Hu et al., "Cysteine–rich Region of Raf–1 Interacts with Activator Domain of Post–translationally Modified Ha–Ras", *J. Biol. Chem.*, 270(51):30274–77 (1995).

Kyriakis et al., "Mitogen Regulation of c–Raf–1 Protein Kinase Activity Toward Mitogen–activated Protein Kinase–kinase", *J. Biol. Chem.*, 268:16009–19 (1993).

Leevers et al., "Requirement for Ras in Raf Activation is Overcome by Targeting Raf to the Plasma Membrane", *Nature*, 369:411–14 (1994).

Luo et al., "Identification of the 14.3.3 ζ Domains Important for Self–association and Raf Binding", 270:23681–687 (1995).

Marble et al., "Peptides Block Ras Function; Potentially Blocks Oncogenic Development", *Cancer Biotech Weekly*, p. 6(1), (1996).

McGeady et al., "The Farnesyl Group of H–Ras Facilitates the Activation of a Soluble Upstream Activator of Mitogen–activated Protein Kinase", *J. Biol. Chem.*, 270:26347–51 (1995).

Nassar et al., "The 2.2 Å Crystal Structure of the Ras–binding Domain of the Serine/Threonine Kinase c–Raf–1 in Complex with Rap 1A and a GTP Analogue", *Nature*, 375:554–60 (1995).

Niehof et al., "A Small Peptide Derived from the Aminoterminus of c–Raf–1 Inhibits c–Raf–1/Ras Binding", *Biochem. and Biophys. Res. Comm.*, 206:46–50 (1995).

(List continued on next page.)

Primary Examiner—Sheela Huff
Attorney, Agent, or Firm—Fish & Richardson, P.C.

[57] **ABSTRACT**

The invention discloses methods of inhibiting direct binding of Ras with Raf and screening methods to identify compounds which inhibit direct binding of Ras to Raf, Raf activation, and cell proliferation.

7 Claims, 8 Drawing Sheets

DOCUMENT-IDENTIFIER: US 5767075 A
TITLE: Inhibiting protein interactions

Detailed Description Text (2):
To facilitate the expression, purification, and solid state immobilization of polypeptides such as Ras or Raf, GST fusion proteins can be made. A chimeric gene encoding a GST fusion protein can be constructed by fusing DNA encoding a peptide or peptide fragment to the DNA encoding the carboxyl terminus of GST (see e.g., Smith et al., 1988, Gene 67:31). The fusion construct, can be transformed into a suitable expression system, e.g., E. coli XA90 in which the expression of the GST fusion protein can be induced with isopropyl-.beta.-D-thiogalactopyranoside (IPTG).

Detailed Description Text (8):
Fragments of Ras which bind Raf (or fragments of Raf which bind Ras) can be made by methods known to those skilled in the art. For example, a DNA fragment which expresses a putative Raf-binding Ras fragment can be fused to GST (as described herein), the fusion protein immobilized by binding to glutathione-SEPHAROSE.TM., and the ability of the fusion protein to bind Raf or a fragment thereof determined.

Detailed Description Text (46):
In another screening method, candidate compounds can be evaluated for anti-proliferative activity by contacting Raf or a Ras-binding fragment of Raf with a candidate compound and determining binding of the candidate compound to the peptide. Raf or Ras-binding fragment of Raf can be immobilized using methods known in the art such as binding a GST-Raf fusion protein to a polymeric bead containing glutathione. Binding of the compound to the Raf peptide is correlated with the ability of the compound to disrupt the signal transduction pathway and thus inhibit cell proliferation.

Detailed Description Text (49):
In another screening method, one of the components of the Ras-Raf binding complex, such as Ras or a Raf-binding fragment of Ras or Raf or a Ras-binding fragment of Raf, is immobilized. Peptides can be immobilized using methods known in the art, such as adsorption onto a plastic microtiter plate or specific binding of a GST-fusion protein to a polymeric bead containing glutathione. For example, GST-Raf(1-257) can be bound to glutathione-SEPHAROSE.TM. beads. The immobilized peptide is then contacted with the labeled peptide to which it binds (Ras in this case) in the presence and absence of a candidate compound. Unbound peptide can then be removed and the complex solubilized and analyzed to determine the amount of bound labeled peptide. A decrease in binding is an indication that the candidate compound inhibits the interaction of Ras with Raf.

Detailed Description Text (51):
A variation of the above-described screening method can be used to screen for another class of candidate compounds which are capable of disrupting a previously-formed Ras-Raf interaction. In this example, a complex comprising Ras or a Raf-binding fragment thereof bound to Raf or a Ras-binding fragment thereof is immobilized as described above and contacted with a candidate compound. The dissolution of the complex by the candidate compound correlates with the ability of the candidate compound to disrupt or inhibit the interaction of Ras with Raf.

Previous Doc Next Doc Go to Doc#

US005582995A

United States Patent [19]

Avruch et al.

[11] Patent Number: 5,582,995

[45] Date of Patent: Dec. 10, 1996

[54] **METHODS OF SCREENING FOR COMPOUNDS WHICH INHIBIT THE DIRECT BINDING OF RAS TO RAF**

[75] Inventors: **Joseph Avruch**, Brookline; **Xian-feng Zhang**, Cambridge, both of Mass.

[73] Assignee: **The General Hospital Corporation**, Boston, Mass.

[21] Appl. No.: **77,256**

[22] Filed: **Jun. 11, 1993**

[51] **Int. Cl.**[6] **G01N 33/53**; G01N 33/574

[52] **U.S. Cl.** **435/71**; 435/7.23; 435/69.1; 435/69.8; 435/69.9; 436/501; 436/64

[58] **Field of Search** 435/7.1, 69.1, 435/69.8, 69.9, 7.23; 436/501, 64

[56] **References Cited**

FOREIGN PATENT DOCUMENTS

WO93/04170 4/1993 WIPO .

OTHER PUBLICATIONS

Fields et al. Nature 340 pp. 245–246 (1989) "A Novel Genetic System to Detect Protein—Protein Interactions".

Luban et al. J. Virol. 66 #8 pp. 5157–5160 (Aug. 1992) "Genetic Assay for Multimerization of Retroviral Gag Polyproteins".

Berg J. B. C. 265 #12 pp. 6513–6516 (1990) "Zinc Fingers and Other Metal–binding Domains".

Mulcahy et al. Nature 313 pp. 241–243 (1985) "Requirement for Gas Proto–Oncogene Function During Serum–Stimulated Growth of NIH NT3 Cells".

Rocquigny et al. Biochem. Biocphys. Res. Comm. 180 #2 pp. 1010–1016 (1991) "First Large Scale Chemical Synthesis of the 72 Amino Acid HIV–1 Nucleocapsid Protein NC p. 7 in an Active Form".

Zhang et al. Nature 364 pp. 308–313 (Jul. 1993) "Normal and Oncogenic p21$^{(a)}$ Proteins Bind to the Amino Terminal Regulatory Domain of c–Raf–1".

Dermer, Bio/Technology, vol. 12 Mar. 1994, p. 320 "Another Anniversary for the War on Cancer".

Plattner et al., "Obstacles to drug development from peptide leads", from *Drug Discovery Technologies*, Ed. by Clark et al., Ellis Harwodd Ltd. (1990), pp. 92–126.

TIG, vol. 10, #2, pp. 45–48 (1994) "The 3Rs of life: Ras, Raf and growth regulation".

Bonner et al.; The complete coding sequence of the human *raf* oncogene and the corresponding structure of the c–*raf*–1 gene; Nucleic Acids Research; 14: 1009–1015 (1986).

Bruder et al.; Serum–, TPA–, and Ras–induced expression from Ap–1/Ets–driven promoters requires Raf–1 kinase; Genes & Development; 6:545–556 (1992).

Chien et al.; The two–hybrid system: A method to identify and clone genes for proteins that interact with a protein of interest; Proc. Natl. Acad. Sci. USA; 88:9578–9582 (1991).

Durfee et al.; The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit; Genes & Development; 7:555–569 (1993).

Guan et al.; Eukaryotic Proteins Expressed in *Escherichia coli*: An Improved Thrombin Cleavage and Purification Procedure of Fusion Proteins with Glutathione S–Transferase; Analytical Biochemistry; 192:262–267.

Heidecker et al.; The Role of Raf–1 Phosphorylation in Signal Transduction; Viral Pathology Section; Laboratory of Viral Carcinogenesis; NCI–Frederick Cancer Research and Development Center, Frederick, Maryland 21702.

Howe et al.; Activation of the MAP Kinase Pathway by the Protein Kinase raf; Cell; 71:335–342 (1992).

Huleihel et al.; Characterization of Murine A–*raf*, a New Oncogene Related to the v–*raf* Oncogene; Molecular and Cellular Biology; 6:2655–2662 (1986).

Itoh et al.; The Post–translational Processing of *rasp*21 Is Critical for Its Stimulation of Mitogen–activated Protein Kinase; The Journal of Biological Chemistry; 268:3025–3028 (1993).

Kitayama et al.; Genetic analysis of the Kirsten–*ras*–revertant 1 gene: Potentiation of its tumor suppressor activity by specific point mutations; Proc. Natl. Acad. Sci. USA; 87:4284–4288 (1990).

Kolch et al.; Raf–1 protein kinase is required for growth of induced NIH/3T3 cells; Nature; 349:426–428 (1991).

Kovacina et al.; Insulin Activates teh Kinase Activity of the Raf–1 Proto–oncogene by Increasing Its Serine Phosphorylation; The Journal of Biological Chemistry; 265:12115–12118 (1990).

Kyriakis et al.; Raf–1 activates MAP kinase—kinase; Nature; 358:417–421 (1992).

Marshall et al.; Characterization of Ras effector mutant interactions with the NF1–GAP related domain; Oncogene; 8:425–431 (1993).

Marshall et al.; Identification of Amino Acid Residues Required for Ras p21 Target Activation; Molecular and Cellular Biology; 11:3997–4004 (1991).

Mizuno et al.; A stimulatory GDP/GTP exchange protein for smg p21 is active on the post–translationally processed form of c–Ki–ras p21 and rhoA p21; Proc. Nat. Acad. Sci. USA; 88:6442–6446 (1991).

Quilliam et al.; Biochemical Characterization of Baculovirus–Expressed *rap*1A/Krev–1 and Its Regulation by GTPase–Activating Proteins; Molecular and Cellular Biology; 10:2901–2908 (1990).

Satoh et al.; Function of Ras as a Molecular Switch in Signal Transduction; The Journal of Biological Chemistry; 267:24149–24152 (1992).

Stanton et al.; Definition of the Human *raf* Amino–Terminal Regulatory Region by Deletion Mutagenesis; Molecular and Cellular Biology; 9:639–647 (1989).

Tsuda et al.; A Protein Kinase Similar to MAP Kinase Activator Acts Downstream of the Raf Kinase in Drosophila; Cell; 72:407–414 (1993).

Valencia et al.; The *ras* Protein Family: Evolutionary Tree and Role of Conserved Amino Acids; Biochemistry; 30:4637–4647 (1991).

Primary Examiner—Lora M. Green

Attorney, Agent, or Firm—Fish & Richardson P.C.

[57] **ABSTRACT**

Disclosed is a method of screening for compounds which inhibit the direct binding of Ras or Raf-binding fragments thereof to Raf or Ras binding fragments thereof.

2 Claims, No Drawings

An assay kit comprising

a receptor-release ligand complex comprising a receptor bound to a monomeric or polymeric release ligand, wherein the receptor is capable of binding to an analyte, wherein the monomeric form of the release ligand binds to the receptor with an association constant of 1% or less of the association constant of the analyte for the receptor, and wherein the release ligand does not detectably compete with analyte for binding to the receptor, wherein the complex is immobilized onto a solid support phase, and

detection means for indicating the presence or amount of receptor or release ligand upon dissociation from the release ligand-receptor complex.

19. The kit of claim 18 wherein the solid phase on which the complex is immobilized comprises a membrane which comprises a reaction field containing an indicator zone, wherein the receptor-release ligand complex is located in the reaction field, and at least part of the detection means is located in the indicator zone.

20. The kit of claim 18 comprising release ligand-receptor complexes for multiple analytes.

21. A method for obtaining a release ligand for use in an assay, wherein the release ligand forms a complex with a receptor which binds an analyte to be detected, comprising

identifying compounds which are structurally similar to the analyte to be detected or which form a part of the analyte,

screening the compounds to identify compounds binding to the receptor, and

identifying the compounds which bind to the receptor in the presence and absence of analyte to select the compounds which bind to the receptor with an association constant of 1% or less of the association constant of the analyte for the receptor, and do not detectably compete with analyte for binding to the receptor.

22. The method of claim 21 wherein the compounds are prepared by synthesizing the analytes with one or more substitutions in the chemical structure.

23. The method of claim 21 wherein the compounds are prepared by isolating the epitope of the analyte bound by the receptor, and modifying the epitope to alter the binding properties.

Previous Doc Next Doc Go to Doc#
First Hit

Generate Collection

L4: Entry 9 of 10 File: DWPI Mar 10, 1995

DERWENT-ACC-NO: 1995-145531
DERWENT-WEEK: 199630

TITLE: Screening for inhibitors or ras-protein partner interactions - using a ras-binding domain coated on scintillation plates and tritium-labelled GTP

PATENT-ASSIGNEE: ELLSTON J M (ELLSI), HUDSON K (HUDSI), ZENECA PHARM CANCER RES DEPT (ZENE)

PRIORITY-DATA: 1995RD-0371034 (February 20, 1995)

Search Selected Search ALL Clear

PATENT-FAMILY:

PUB-NO	PUB-DATE	LANGUAGE	PAGES	MAIN-IPC
RD 371034 A	March 10, 1995	E	002	C12N000/00

APPLICATION-DATA:

PUB-NO	APPL-DATE	APPL-NO	DESCRIPTOR
RD 371034A	February 20, 1995	1995RD-0371034	

INT-CL (IPC): C12 N 0/00

ABSTRACTED-PUB-NO: RD 371034A
BASIC-ABSTRACT:

Screening method is disclosed for identifying inhibitors of ras-protein partners interactions. A ras-binding domain of c-raf-1 is immobilised on microtitre plates coated with scintillant (flash plates and then ras pre-loaded with tritium-radiolabelled GTP is added. Binding of ras-GTP to immobilised raf juxtaposes the radiolabel to the scintillant, generating a signal. The signal is reduced to background through (i) loading ras with radiolabelled GDP instead of GTP, which gives non-active ras protein, (ii) addn. of peptide inhibitors such as ras 67-44, (iii) addn. of novel ras/raf inhibitors.

USE - The method can be used for the high throughput screening of potential anti-cancer therapeutic agents.

ABSTRACTED-PUB-NO: RD 371034A
EQUIVALENT-ABSTRACTS:

CHOSEN-DRAWING: Dwg.0/0

DERWENT-CLASS: B04 D16 K08
CPI-CODES: B04-B03B; B04-N02; B05-A04; B11-C07B2; B12-K04A; D05-H09; D05-H10; K08-

x;

Previous Doc Next Doc Go to Doc#

First Hit Previous Doc Next Doc Go to Doc#

Generate Collection Print

L9: Entry 10 of 15 File: DWPI Mar 10, 1995

DERWENT-ACC-NO: 1995-145531
DERWENT-WEEK: 199630

TITLE: Screening for inhibitors or ras-protein partner interactions - using a ras-binding domain coated on scintillation plates and tritium-labelled GTP

PRIORITY-DATA: 1995RD-0371034 (February 20, 1995)

Search Selected Search ALL Clear

PATENT-FAMILY:

PUB-NO	PUB-DATE	LANGUAGE	PAGES	MAIN-IPC
RD 371034 A	March 10, 1995	E	002	C12N000/00

INT-CL (IPC): C12 N 0/00

ABSTRACTED-PUB-NO: RD 371034A
BASIC-ABSTRACT:

Screening method is disclosed for identifying inhibitors of ras-protein partners interactions. A ras-binding domain of c-raf-1 is immobilised on microtitre plates coated with scintillant (flash plates and then ras pre-loaded with tritium-radiolabelled GTP is added. Binding of ras-GTP to immobilised raf juxtaposes the radiolabel to the scintillant, generating a signal. The signal is reduced to background through (i) loading ras with radiolabelled GDP instead of GTP, which gives non-active ras protein, (ii) addn. of peptide inhibitors such as ras 67-44, (iii) addn. of novel ras/raf inhibitors.

USE - The method can be used for the high throughput screening of potential anti-cancer therapeutic agents.

ABSTRACTED-PUB-NO: RD 371034A
EQUIVALENT-ABSTRACTS:

CHOSEN-DRAWING: Dwg.0/0

Previous Doc Next Doc Go to Doc#

Previous Doc Next Doc Go to Doc#
First Hit

Generate Collection

L3: Entry 49 of 173 File: PGPB Jan 23, 2003

DOCUMENT-IDENTIFIER: US 20030017519 A1
TITLE: Electrochemical enzyme assay

Abstract Paragraph:
A diagnostic kit, method, and apparatus for electrochemically determining the presence or concentration of an analyte in a sample. A mixture is formed which includes the sample, an enzyme acceptor polypeptide, an enzyme donor polypeptide, and a labeled substrate. The enzyme donor polypeptide is capable of combining with the enzyme acceptor polypeptide to form an active enzyme complex. The formation of such the active enzyme complex is responsive to the presence or concentration of the analyte in the fluid sample. The active enzyme hydrolyzes the labeled substrate, resulting in the generation of an electroactive label, which can then be oxidized at the surface of an electrode. A current resulting from the oxidation of the electroactive compound can be measured and correlated to the concentration of the analyte in the sample.

Previous Doc Next Doc Go to Doc#

Previous Doc Next Doc Go to Doc#
First Hit Fwd Refs

Generate Collection

L6: Entry 9 of 9 File: USPT May 15, 2001

DOCUMENT-IDENTIFIER: US 6232081 B1
TITLE: Method for the detection of NF-.kappa.B regulatory factors

Detailed Description Text (152):
Next, it was determined whether Cln/Cdc28, present in small amounts in the ubiquitination reaction, is also required for additional steps in the ubiquitination process (e.g., to phosphorylate the ubiquitination machinery). This was accomplished by treating bacterial Sic1 with Cln2/Gst-Cdc28 complexes immobilized on GSH-SEPHAROSE beads, removing the complexes from the beads prior to use in ubiquitination reactions, and determining whether the complexes were free of soluble kinase by immunoblotting with anti-HA antibodies (See, FIG. 3D, lane 3). These results indicated that Sic1 phosphorylated in this manner was also efficiently ubiquitinated (See, FIG. 3D, lane 9). Thus, these data indicated that Sic1 phosphorylation constitutes the primary requirement of Cln/Cdc28 kinases in Sic1 ubiquitination in the in vitro reaction.

Previous Doc Next Doc Go to Doc#

Previous Doc Next Doc Go to Doc#
First Hit Fwd Refs

Generate Collection

L6: Entry 8 of 9 File: USPT Jun 3, 2003

DOCUMENT-IDENTIFIER: US 6573094 B1
TITLE: F-box genes and proteins

Detailed Description Text (148):
Next, it was determined whether Cln/Cdc28, present in small amounts in the ubiquitination reaction, is also required for additional steps in the ubiquitination process (e.g, to phosphorylate the ubiquitination machinery). This was accomplished by treating bacterial Sic1 with Cln2/Gst-Cdc28 complexes immobilized on GSH-Sepharose beads, removing the complexes from the beads prior to use in ubiquitination reactions, and determining whether the complexes were free of soluble kinase by immunoblotting with anti-HA antibodies (See, FIG. 3D, lane 3). These results indicated that Sic1 phosphorylated in this manner was also efficiently ubiquitinated (See, FIG. 3D, lane 9). Thus, these data indicated that Sic1 phosphorylation constitutes the primary requirement of Cln/Cdc28 kinases in Sic1 ubiquitination in the in vitro reaction.

Previous Doc Next Doc Go to Doc#

DERWENT-ACC-NO: 1995-145531
DERWENT-WEEK: 199630

TITLE: Screening for inhibitors or ras-protein partner interactions - using a ras-binding domain coated on scintillation plates and tritium-labelled GTP

PATENT-ASSIGNEE: ELLSTON J M (ELLSI), HUDSON K (HUDSI), ZENECA PHARM CANCER RES DEPT (ZENE)

PRIORITY-DATA: 1995RD-0371034 (February 20, 1995)

Search Selected | Search ALL | Clear

PATENT-FAMILY:

	PUB-NO	PUB-DATE	LANGUAGE	PAGES	MAIN-IPC
☐	RD 371034 A	March 10, 1995	E	002	C12N000/00

APPLICATION-DATA:

PUB-NO	APPL-DATE	APPL-NO	DESCRIPTOR
RD 371034A	February 20, 1995	1995RD-0371034	

INT-CL (IPC): C12 N 0/00

ABSTRACTED-PUB-NO: RD 371034A
BASIC-ABSTRACT:

Screening method is disclosed for identifying inhibitors of ras-protein partners interactions. A ras-binding domain of c-raf-1 is immobilised on microtitre plates coated with scintillant (flash plates and then ras pre-loaded with tritium-radiolabelled GTP is added. Binding of ras-GTP to immobilised raf juxtaposes the radiolabel to the scintillant, generating a signal. The signal is reduced to background through (i) loading ras with radiolabelled GDP instead of GTP, which gives non-active ras protein, (ii) addn. of peptide inhibitors such as ras 67-44, (iii) addn. of novel ras/raf inhibitors.

USE - The method can be used for the high throughput screening of potential anti-cancer therapeutic agents.

ABSTRACTED-PUB-NO: RD 371034A
EQUIVALENT-ABSTRACTS:

CHOSEN-DRAWING: Dwg.0/0

DERWENT-CLASS: B04 D16 K08
CPI-CODES: B04-B03B; B04-N02; B05-A04; B11-C07B2; B12-K04A; D05-H09; D05-H10; K08-X;